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THE ELECTRICAL AND MECHANICAL EVENTS OF NEURO-MUSCULAR TRANSMISSION IN THE COCKROACH, *PERIPLANETA AMERICANA* (L.)*

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(With Plate 1 and Four Text-figures)

Though the mechanical activity of insect muscle has been studied by many workers (notably Heidermanns, 1931; Solf, 1931; Kraemer, 1932; Cremer, 1935), and insect-muscle potentials have been recorded by Rijlant (1932) and Pringle (1939), no attempt has been made to examine the precise relation between motor-nerve impulse, muscle potential, and muscle contraction in this group. The purpose of this account is to examine the electrical and mechanical signs of neuro-muscular events in the cockroach, and to evaluate them in the light of current concepts of neuro-muscular transmission in Crustacea and invertebrates.

METHOD

Adults of the American cockroach, *Periplaneta americana*, were used throughout. Though the same results were obtained with both sexes, males were preferred owing to the relative absence of fat within the thorax. The insects were decapitated and pinned ventral side up in a shallow Petri dish which had been half filled with wax. The metathoracic legs were widely spread and held in a position of retraction by means of pins. The metathoracic ganglion, nerves, and the extrinsic leg muscles were exposed by removal of the ventral cuticle, surrounding fat and tracheae (Pl. 1). Nerve 3 (Pringle, 1939) was prepared for placement of stimulating electrodes by cutting branch *B* which supplies muscles within the coxa, and by tracing branch *A* to the *tergal muscles of the trochantin* (Carbonell, 1947). The latter consist of three long (5–6 mm.) and narrow muscles which originate from the anterior part of the metathoracic tergum and pass downward and backward to a common insertion on the median end of the trochantin close to the anterior median end of the coxa. From the ventral approach they are overlapped by the broad episternal pro-motor muscle, which must be removed in part before they can be completely exposed. For some studies the whole group was employed, though the effect of stimulation of nerve 3 could be limited to the *second tergal muscle of the trochantin* (muscle 162 of Carbonell) by careful section of small branches from nerve 3*A* which pass to the other muscles.

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This muscle, which will be designated as muscle 162, is the most medial member of the group. It was the subject of most of the experiments reported below.

Reflex responses were prevented by cutting other nerves leaving the ganglion, and by crushing the ganglion with forceps. Nerve 3 was allowed to remain attached to the remains of the ganglion, since its small size made handling difficult and survival short after it had been severed.

Sufficient insect Ringer was added so as to cover the wax at the bottom of the dish. Seepage of Ringer up through the cut surfaces of the cockroach kept the preparation in excellent condition for several hours with little or no addition of fluid to the surface of the muscle. Stimulation of nerve 3 was accomplished while it lay just below the surface film of liquid collecting between muscle and ganglion. If raised above the liquid the nerve dried within a few minutes, while immersion in a pool of mineral oil caused shrinkage and deterioration.

Stimulation was accomplished by fine tapered and hooked silver electrodes, which were brought into position below the nerve by means of a manipulator (Pl. 1). Muscle potentials were recorded from similar electrodes placed directly on the exposed surface of the muscle. For monopolar recording, an 'indifferent' electrode was obtained by placing a ring of silver wire, 5 cm. in diameter, in the saline surrounding the animal.

The onset of contraction was detected by a fine glass stylus placed on the muscle surface near the electrodes used to record potential change. The stylus was inserted in the needle socket of a piezo-electric pick-up of the type ordinarily used in phonograph play-back equipment. The pick-up was connected to one of the amplifiers and cathode-ray deflexion system. It served as an extremely sensitive indicator of rapid and small movements because of the high gain of the amplification used. It was used only to determine the onset of mechanical change in the muscle, hence its linearity and frequency characteristic are of no consequence.

Recording equipment consisted of two Grass P3 resistance-capacity coupled amplifiers with a flat response up to 15 kcyc./sec. After further amplification, potential changes provided the Y deflexion for two beams of a du Mont 5SP11 cathode-ray tube. The X deflexion of both beams was accomplished by the same sweep circuit throughout. The stimulus was a square pulse of variable duration triggered by the sweep pulse. A stimulus to nerve 3 of 1–2 V. (at the stimulator) and 0·2 msec. duration ordinarily caused a stepless response of muscle 162.

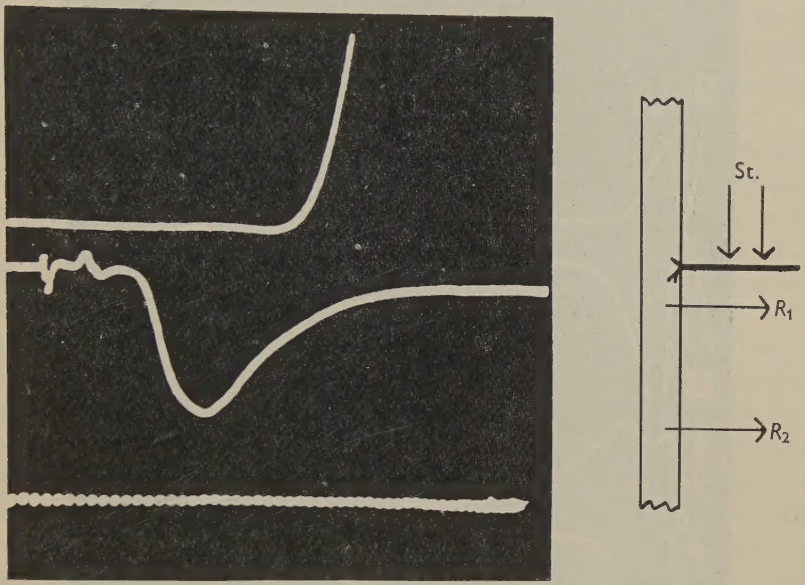
All experiments were carried out at room temperature.

EXPERIMENTAL RESULTS

Temporal relations of the muscle potential. The sequence of electrical and mechanical events following indirect stimulation of muscle 162 are shown in Text-fig. 1. Potential changes on the surface of the muscle were recorded between electrode R_1 placed in the middle of the muscle opposite the point of nerve entrance, and electrode R_2 2 mm. distant on the anterior end of the muscle.

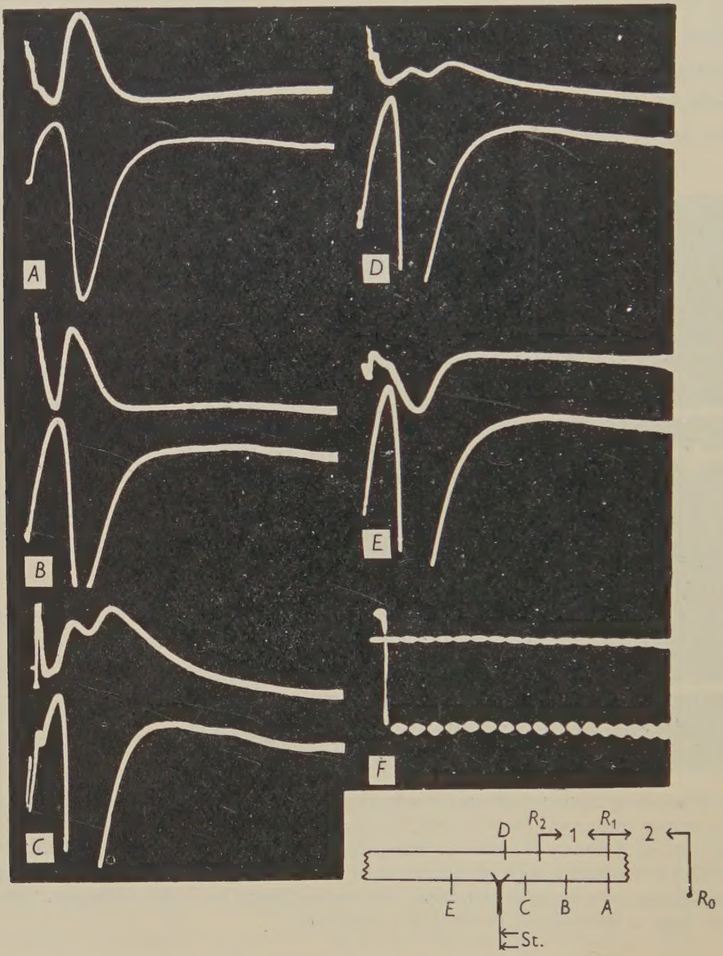
Following the stimulus artifact by 0·5 msec. is a small upward deflexion produced by the action potential in the motor nerve as the latter enters the muscle substance.

The muscle potential appears as a downward deflexion (positivity of R_1) about 1·0–1·3 msec. after the nerve spike. It reaches a maximum in 1·3 msec., falling to about one-half of its peak value in a similar period. This is followed by a slower decline, leaving in some cases a small potential which persists throughout the contraction. The major part of the muscle potential occupies 4–5 msec., and muscle shortening begins abruptly during the latter part of its declining phase. The latent period (from onset of muscle potential to onset of contraction) lasts 2·5–3·2 msec. Using mechanical recording, Solf (1931) gives a latent period of 9·0 msec. for the same muscle in *Decticus verrucivorus*. The shorter value given here is undoubtedly due to the relatively inertia-free method of registration, and the fact that nerve conduction and end-plate delay are not included in the measurement. In some piezo-electric recordings of the muscle contraction, a small degree of relaxation appears to precede the active shortening. This may be an example of latent relaxation, though it could be due to muscle movement taking place at some point distant from the recording stylus.



Text-fig. 1. Recording of electrical and mechanical changes following indirect stimulation of muscle 162. The upper trace records movement of the muscle at point R_1, and the middle trace records potential change between R_1 and R_2. The first rectangular deflexion is the stimulus artifact. This is followed by the small upward deflexion produced by the nerve-action potential. The muscle potential follows as a large monophasic downward (positive at R_1) deflexion. The brightness of the lower trace is modulated at 0·2 msec. intervals.

Sign of the muscle potential. When the muscle potential is recorded between two electrodes on the muscle surface, that nearest to the point of nerve entrance (Text-fig. 1, R_1) becomes positive to an electrode (R_2) placed at either end. Under these circumstances the nerve-action potential has the usual negative sign. In most cases active muscle becomes positive to an 'indifferent' electrode. In the experiment recorded in Text-fig. 2 an active electrode (R_1) on the muscle is pitted against an electrode (R_0) in contact with the saline surrounding the roach. The lower tracing records the positive potential of R_1 against R_0 as the former is moved to various points along the muscle. The maximum deflexion is produced when R_1 is on the middle of the muscle opposite the point of nerve entrance. R_1 is also pitted against another electrode R_2 also on the muscle surface. R_1 and R_2 are separated by a constant distance of 2 mm. as the pair is moved together to different points on the muscle surface. It can be seen that the R_1R_2 potential reaches a minimum and changes sign when the pair is near the point of nerve entry. This shows that a fairly



Text-fig. 2. Recording of potential change between bipolar (R_1R_2, upper trace) and monopolar (R_1R_0, lower trace) electrodes, as electrode pair R_1 and R_2 is moved to different points on the surface of muscle 162. R_0 is in saline surrounding the preparation. Each record was made with the common electrode, R_1 on the correspondingly lettered point on the muscle. R_1 and R_2 were kept at a constant distance apart of 2·0 mm. F, base-line interrupted at 1·0 msec. intervals. Calibration deflexion on lower beam produced by 2·0 mV. For full explanation see text.

steep potential gradient develops along the muscle surface during activity, the surface near the point of nerve entry becoming positive to any other point on the surface, which is in turn positive with respect to the 'indifferent' electrode.

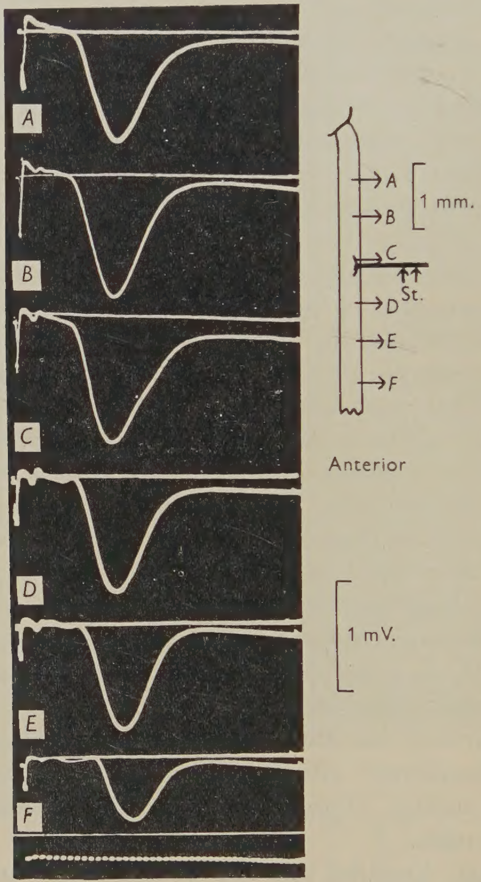
Attempts were made to remove the nerve and muscle entirely from the body. The handling and injury caused by this procedure resulted in a rapid loss of irritability, and it was impractical to cut the muscle away from its origin and insertion on the cuticle. In one experiment both nerve and muscle were removed from the insect leaving some of the cuticle attached to the origin and insertion. This preparation was placed in a shallow dish containing saline and an indifferent electrode. On stimulation of the nerve, the muscle electrode became negative with respect to the saline electrode, both nerve spike and muscle potential appearing as upward deflexions. When two electrodes were placed on the muscle surface of this preparation the central region became positive to either end though the nerve spike retained its negative sign.

Insertion of an electrode tip into the muscle substance made no difference to the recorded sign of the potential, which increased up to a point, only to decrease as the electrode passed right through the muscle. Placement of an electrode on the cut ends of a severed muscle did not give satisfactory results owing to rapid loss of irritability. In one or two cases where a few observations could be made, the surface electrode became positive to the cut surface during activity.

Though active muscle *in situ* was most frequently positive, the sign of the muscle potential was often quite capricious. In one experiment the anterior part of the muscle became positive to a saline electrode while the posterior part became negative. Wiersma & Wright (1947) noted that many active crustacean muscles developed a positive potential, and suggested that in these cases innervation of each fibre from one side or from within the muscle substance made it impossible to bring a surface electrode into contact with the active region. They concluded that the external cuticle afforded a better electrical connexion through the muscle insertion with the cathodally depolarized muscle surfaces than did an electrode placed on the body of the muscle, hence a distant or 'indifferent' electrode could become negative to an electrode placed on the muscle surface. It seems probable that the same explanation applies to insect-muscle potentials.

Form of the potential. Provided that not more than one muscle was excited by motor-nerve stimulation, the muscle potential invariably took a monophasic form. This occurred irrespective of stimulus frequency, interelectrode distance, or form of recording (monopolar or bipolar). At first it was thought that the monophasic curve obtained with bipolar recording (Text-fig. 1) was due entirely to the local or non-propagated nature of the muscle potential. Though this was partly responsible, observation of a muscle contracting rhythmically under stroboscopic illumination shows that all parts of the muscle participate in the shortening process. Therefore, excitation must be propagated throughout the muscle, whether it be through the agency of nerve or muscle fibres.

Recordings were made of the time relations of stimulus artifact, nerve spike, and muscle potential at an electrode placed at measured distances along the muscle and pitted against a saline electrode (Text-fig. 3). Comparison of the oscillograms shows that the time interval between shock artifact and nerve spike remains constant at all points on the muscle, though the nerve potential becomes vanishingly small as the exploratory electrode moves away from the point where the nerve is seen to disappear within the muscle substance. This suggests that the nerve potential originates from the motor nerve at this point, and that spike potentials in motor-nerve branches approaching their terminations cannot be recorded with a surface electrode. Therefore, the neuro-muscular delay cannot be measured directly, though it must have some value less than 1·0–1·3 msec., the interval between nerve spike and muscle potential.



Text-fig. 3. Potential changes registered at different points on muscle 162 following indirect stimulation. An active electrode was placed at measured points along the muscle as indicated by letters. The travelling muscle electrode was pitted against an indifferent (saline) electrode. The records are lettered to correspond with electrode positions. Zero potential difference is indicated by the horizontal line in each record. The lower trace is broken at intervals of 0·2 msec. For details see text.

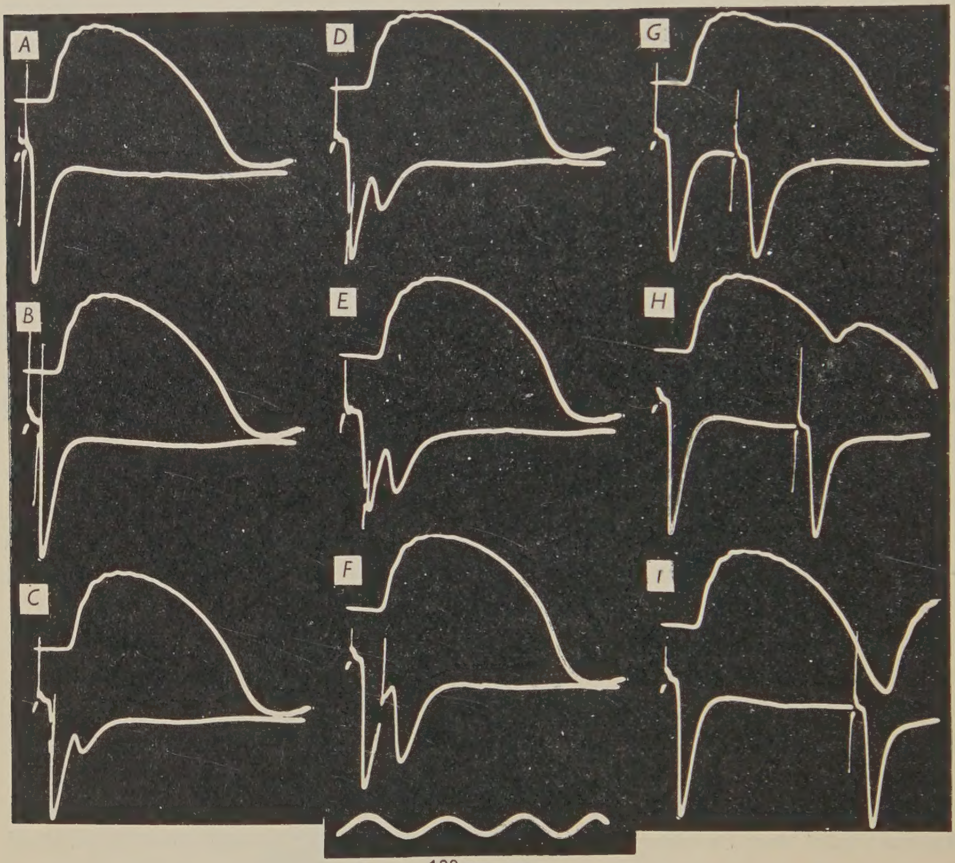
As a single exploratory electrode is moved from the point of nerve entrance toward either end of the muscle (Text-fig. 3), the muscle potential becomes reduced in height and shows a delay in onset. Comparison of oscillograms *C* and *F* in Text-fig. 3 shows that the muscle potential begins 0·4–0·5 msec. later in *F*, which was recorded 2·3 mm. distant from *C*. Though measurements over these short distances have a large error, this would give a rate of spread of the muscle potential of 5–6 mm./msec. Since the duration of the muscle potential is 4–5 msec., its wavelength would be 20–30 mm. or 8–10 times the possible interelectrode distance for two electrodes placed on one-half of the muscle (Text-fig. 1). Therefore, the onset of potential change under the distal electrode of a pair on the muscle is only slightly displaced relative to change under the proximal electrode, and the monophasic potential is due almost entirely to the potential gradient developed along the muscle, owing little or nothing to propagation.

The motor unit and summation. Providing the response to a single indirect stimulus was limited to a single muscle, electrical and mechanical responses always occurred together and showed no stepwise increase with changes in stimulus strength. It is concluded that the response of muscle 162 was due to stimulation of a single motor fibre which innervates all the muscle fibres. This has been demonstrated by Pringle (1939) for other muscles in the cockroach. He also showed that each muscle was supplied by a 'quick' fibre which was responsible for a muscle potential and maximal twitch showing no summation at any stimulus frequency (up to 150 per sec.), and a 'slow' fibre which operated only through summation and caused a gradual shortening of the muscle on repetitive stimulation.

A few experiments were carried out with pairs of indirect stimuli separated by a variable interval (Text-fig. 4). When the stimulus interval was less than 1·8 msec. (Text-fig. 4, *A*, *B*), the second shock evoked no electrical response. When it was lengthened to 1·9–2·0 msec., the muscle potential due to the second shock appeared as a small hump on the decaying phase of the first muscle potential (Text-fig. 4, *C*). Further separation of the stimuli up to a 4·0 msec. interval caused rapid growth of the second potential. Slower growth continued up to a stimulus interval of 10 msec. when the second potential approached the first potential in size (Text-fig. 4, *G*). Since the whole muscle appears to be innervated by a single 'quick' fibre, changes in size of the muscle potential must be due to changes at the neuro-muscular junctions. The interval of 2–10 msec. occupied by the recovery of the muscle potential could then be occupied by the progressive return to activity of individual neuro-muscular junctions from a refractory residue. The whole picture is one of depression rather than summation.

The mechanical response of the muscle to two stimuli runs parallel to the electrical response. It must be noted that registration of shortening by means of the piezo-electric pick-up introduces serious distortion during the later phases of contraction. This is seen as a dip below the base-line (Text-fig. 4, upper trace in each record). In addition, the amplifier coupling prevents registration of any sustained shortening so that the mechanical records must be viewed with some reservations. However, no prolongation of the muscle contraction is evident until the stimulus interval approaches 5·0 msec. (Text-fig. 4, *F*). While the stimulus interval lies between 5·0 and 10·0 msec. the twitches remain fused, though when allowance is made for the distortion mentioned above, the second peak appears to be much lower than the first. With an interval of 10·0–20·0 msec. the twitches separate (Text-fig. 4, *G*, *H*), and no mechanical summation is evident.

Direct stimulation of the muscle. Placement of stimulating electrodes directly on muscle 162 caused an electrical response with similar threshold and latency to that produced by stimulation of nerve 3, and there is every reason to suspect that the response was due to stimulation of small branches of the motor nerve within the muscle substance.



Text-fig. 4. The effect of a pair of indirect stimuli upon the electrical and mechanical responses of muscle 162. The method of recording is similar to that used in Text-fig. 1, except that the muscle potential was recorded against an inactive electrode. The time calibration (100 cyc./sec.) applies to electrical and to mechanical response. The first of a pair of 150 μsec. liminal stimuli was applied at the beginning of the sweep in each record. The stimulus artifact is visible as a small gap at the extreme left of the lower trace. The interval in milliseconds at which the second stimulus (second gap in lower trace) followed the first was lengthened in each recording, and is as follows: *A*, 0·8; *B*, 1·8; *C*, 2·0; *D*, 2·3; *E*, 2·8; *F*, 4·4; *G*, 10·0; *H*, 18·0; *I*, 25·0.

Curare (b tubocurarine Squibb, 10^{-3}) failed to alter the indirect response of muscle 162. Therefore, in order to eliminate the motor nerve as a source of muscle stimulation, nerve 3 was severed surgically in a number of adult male cockroaches. After some practice, this could be done through a small incision next to the ganglion with no damage to the latter or to other nerves. Approximately 90% of the insects survived, and appeared to be entirely normal except for a motor defect in one metathoracic leg. They were kept in a container with food and water, and individuals were withdrawn at intervals for experimentation. Muscle 162 was exposed on the denervated and on the intact side of each insect, and both muscles were subjected to direct electrical stimulation.

For 3–5 days after nerve section, there was little difference in the response to electrical stimulation of denervated and normal muscles. Quite abruptly at the end of this period, the denervated muscles failed to show any response whatever, and a similar situation existed up to 14 days after nerve section, which is the longest period over which operated insects have been followed. Stimuli up to 50 V. (at the stimulator) and 1·0 msec. in duration failed to stimulate the denervated muscles, though a stimulus of 1–3 V. and 0·1–0·2 msec. duration elicited a maximal response from the intact muscle 162 in the same insects. With the thought that perhaps the chronaxie of denervated muscle was very different from that of nerve, direct current with a rapid rate of rise was applied through electrodes placed on the muscle surface. Though the maximum current strength used caused violent electrolysis at the electrode site and marked contraction of normal muscles in other parts of the body of the roach, there was no detectable response in the denervated muscles. The preparations were examined closely through a binocular dissecting microscope during stimulation, and a check was made by placing the piezo-electric pick-up close to the point of stimulation. In not one out of twelve successfully denervated muscles could any movement be attributed to the electrical stimulus.

There appears to be no published work on the structural changes in insect muscle and nerve after motor-nerve section, and a histological study is in progress. However, within 14 days of nerve section, fresh denervated muscle showed no gross or microscopic changes in colour, size, appearance, or presence of striations. Visible traces of the peripheral nerve stump were lost after 3 days. It seems to be established that cockroach muscle becomes electrically inexcitable after denervation. This suggests that the muscle can be excited only through the agency of the motor-nerve impulse.

DISCUSSION

Potential changes in crustacean muscles are similar in many ways to those described for insects. In addition to reporting that muscle potentials in *Cambarus* had a positive sign, Wiersma & Wright (1947) noted that they were generally monophasic. These authors conclude that the recorded potential represented the sum of local muscle potentials developed at many innervation sites along the muscle fibres. van Harreveld (1939) concluded that crustacean muscle fibres could not be directly excited and that a muscle conduction process was absent. From experiments on neuro-muscular transmission in various marine crabs and fresh-water crayfish, Katz & Kuffler (1946) concluded that single stimuli applied directly or through the motor nerve caused local graded electrical and mechanical responses in crustacean muscle. These negative local potentials were analogous to the end-plate potential of vertebrate muscle and readily summed on repetitive stimulation to produce a potential several times the unit height, from which propagated muscle action potentials took off to produce total muscle twitches.

The experiments reported in this paper suggest a similar conclusion to that reached by van Harreveld (1939) and Wiersma & Wright (1947), namely, that in arthropod muscles conduction is accomplished over nervous rather than muscular pathways. Observations which bear out this conclusion are:

(1) The presence of only one form of quick fibre muscle potential at a variety of stimulus strengths and frequencies. No evidence of a transition from local potential into propagated spike, or of local potential summation as reported by Katz & Kuffler (1946).

(2) A marked potential gradient developed during excitation between the muscle surface near the point of nerve entry, and the muscle surface at either end. This suggests greater depolarization in those regions of the muscle where motor endings might be expected to be most dense. The monophasic potential recorded from two electrodes on the muscle surface is due to this gradient rather than to later arrival of excitation at the ends of the muscle.

(3) Rapid spread (4–6 m./sec.) of excitation in normal muscle from the point of nerve entry toward either end. All parts of the muscle appear to be involved within 0·5 msec. In contrast, there is a complete loss of muscle excitability to electrical stimulation after the motor nerve has been allowed to degenerate.

If this evidence is accepted, the muscle potential in the cockroach is analogous to the local (end-plate) potential of vertebrate and crustacean muscle, the potential change recorded at the muscle surface being the sum of local potentials developed simultaneously in several fibres and sequentially at several points on each fibre. At this point the similarity ends, since in normal vertebrate muscle the local potential invariably grows into a muscle-propagated wave of depolarization, in crustacean muscle it may (Katz & Kuffler, 1946) or may not (Wiersma & Wright, 1947) develop into muscle-propagated excitation, while in insects it remains as a large number of local responses, excitation and propagation throughout the entire muscle being accomplished by the motor nerve. Since curare prevents the development of local into conducted responses in vertebrates, its lack of action on cockroach, and in other arthropod neuro-muscle preparations, could be due to the absence of muscle conduction in arthropods.

It should be pointed out that although the muscle potential appears to be the sum of local responses throughout the muscle, the response of muscle 162 to single indirect stimuli is strictly all-or-none. Hence an impulse in the motor nerve must reach every part of the muscle capable of depolarization, giving a total electrical and mechanical response as far as the quick fibre is concerned. The lack of summation and reduced response to the second of a pair of stimuli indicates relative refractoriness at motor endings in the muscle for 2–10 msec. It is not clear whether this is due to reduced responses at all the innervation points, or to failure of a certain percentage of endings to respond to the second nerve impulse.

Attempts to examine the structural relations of motor nerve and muscle fibres in muscle 162 have not met with much success. Methylene blue and Golgi methods have not differentiated the finer nerve branches, though profusely branched segments of nerve have been seen in one or two teased preparations. However, many early workers have noted the presence of several motor endings or Doyere cones on each insect muscle fibre. Marcu (1929) records 12 and 20 Doyere cones on a millimetre length of thoracic muscle fibre from *Geotrupes stercorarius* and from *Musca domestica* respectively. This author describes each Doyere cone as an abrupt and brush-like branching of each small division of the motor nerve as it reaches the surface of the sarcolemma. The extremely fine fibres composing the brush penetrate the sarcolemma and pass into the sarcoplasm among the myofibrillae. He noted that the number of Doyere cones varied in different regions of the same muscle fibre, though in some places they were so closely packed that the 'brushes' overlapped and intermingled. In thoracic muscle of *Phyllodromia germanica*, Marcu noted that compact Doyere cones were absent, though the motor nerve branched profusely, and its terminal fibres made contact with the sarcolemma of each muscle fibre at many points. Foettinger (1880) made an examination of muscle fibres from several species of insects which had been rapidly fixed while in a state of partial contraction. He concluded that local contractions could occur only in the vicinity of motor endings. This anatomical picture makes it difficult to see how propagated excitation in insect muscle is either necessary or possible, and the motor nerve appears to provide the sole means of conduction.

Some light on the cause of the positive potential in insect muscle is shed by the observations of Marcu (1929). He noted that Doyere cones were distributed mainly along one side of each muscle fibre, and in one of his figures consisting of a cross-section of eight muscle fibres, the Doyere cones are represented as lying only upon the inner surface of each muscle fibre of the group. This is in accord with the explanation proposed by Wiersma & Wright (1947) for positive muscle potentials in Crustacea. They suggested that the locally active and cathodally polarized muscle surfaces were arranged in such a manner as to make better electrical connexion with the muscle insertion and external cuticle than with the overlying muscle surface. In a cylindrical muscle this could be realized if the asymmetrically innervated muscle fibres were grouped in cylindrical bundles with the motor nerve running in the centre of the bundle and innervating each fibre at several points along its inner surface. The catelectrotonus developed on excitation would be limited in the absence of muscle conduction to the inner side of each fibre, and the negativity developed in the core of the bundle might cause inward current flow from the muscle insertions if the fibres were closely packed. Hence, the muscle surface would become a source of current which would flow towards muscle origin and insertion.

The complete lack of response of denervated muscle to any form of electrical stimulation remains very puzzling. Absence of propagated excitation in denervated muscle is not unexpected if the motor nerve normally performs this function, but the absence of a local response of any kind cannot be explained. Either the muscle surface has such high electrical resistance that externally applied current fails to reach the depolarizable regions of muscle fibres arranged as described in the last paragraph, or cockroach muscle is electrically inexcitable. In order to examine the last possibility studies on the chemical sensitivity of denervated muscle are in preparation. No way can be seen by which the first possibility could be tested.

SUMMARY

1. A nerve-muscle preparation in the metathorax of the cockroach is described. It consists of the second tergal muscle of the trochantin (muscle 162 of Carbonell) innervated by a branch of nerve 3 *A*. Electrical changes are recorded from electrodes on the muscle surface, and the onset of contraction is registered by the stylus of a piezo-electric pick-up.

2. With low (3–5 per sec.) stimulation rates at room temperature the neuromuscular delay is less than 1·2 msec., and the latent period of contraction about 3·0 msec. The muscle potential is 4–5 msec. in duration, positive in sign at the muscle surface, and monophasic in form with either monopolar or bipolar recording. During excitation a potential gradient develops along the muscle, the greatest positivity being in the middle near the point of nerve entry.

3. Neither electrical nor mechanical response show gradations with changes in stimulus strength or frequency. No facilitation is evident, and the response appears to be due to stimulation of a single quick motor nerve fibre.

4. In order to study the effects of direct stimulation nerve 3 was sectioned and allowed to degenerate. All trace of the peripheral nerve stump was lost after 3 days, when the muscle became completely inexcitable to all forms of electrical stimulation. There were no gross structural changes which would account for this loss of excitability.

5. It is concluded that the recorded muscle potential in the cockroach is analogous to the vertebrate end-plate potential, being the sum of local muscle potentials developing simultaneously in several fibres, and sequentially at several innervation points along the same fibre. Conduction within the muscle is carried out entirely by motor nerve fibres.

6. Possible causes of the positive sign of the muscle during activity are discussed.

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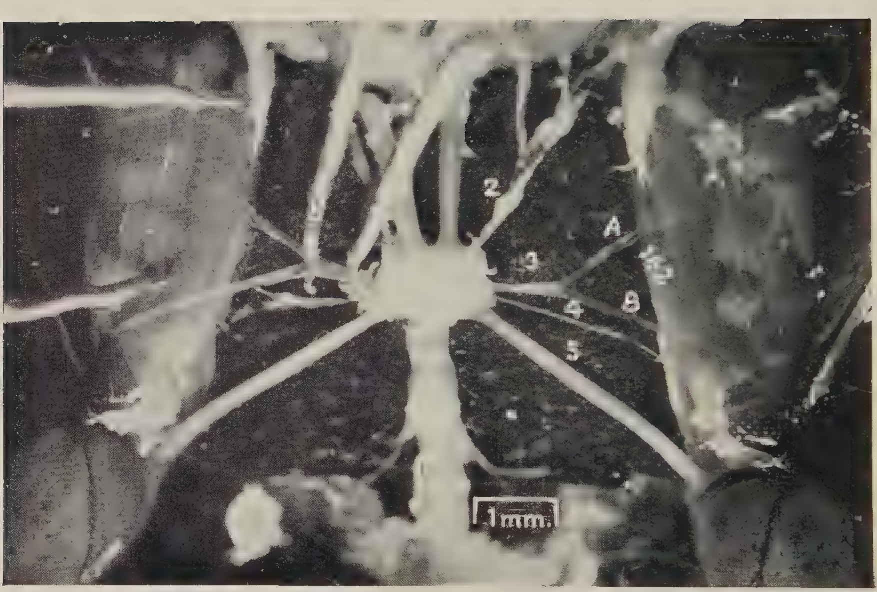

ROEDER AND WEIANT—NEURO-MUSCULAR TRANSMISSION IN THE COCKROACH

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EXPLANATION OF PLATE 1

Fresh dissection of the metathoracic ganglion, nerves and tergal muscles of the trochantin. Nerves are numbered according to the usage of Pringle (1939). On the left stimulating electrodes have been placed below nerve 3 and recording electrodes rest on the tergal muscles of the trochantin. Muscle 162 lies just to the right of the electrode tips. During experimentation branch *B* and several smaller branches of nerve 3 would be severed.

THE MECHANICS OF THE BLOOD VASCULAR SYSTEM OF *ASCIDIELLA ASPERSA*

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(With Ten Text-figures)

INTRODUCTION

The regular change in the direction of the heart beat and resulting reversal of the circulation in tunicates is well known. Various suggestions as to the cause of this have been put forward from time to time, the most important being the so-called 'back-pressure' theory. This theory was put forward some time ago and has received considerable criticism. The present paper provides experimental and theoretical evidence in support of this theory.

PREVIOUS WORK

The reversal of the heart beat in tunicates has attracted considerable attention, and an early theory to account for the reversal of the heart was put forward by Van Hasselt (1824), based on the idea that in salps the blood corpuscles blocked the finer channels of the blood system, the heart reversing to relieve the congestion. Another theory suggested that the spongy tissue of the pyloric gland in ascidians offered a point of resistance in the circulation (Hancock, 1868). Many other people (Herdman, 1899; Herdman, 1924; Pizon, 1899, 1900; Ritter, 1893; Roule, 1885; Wagner, 1866) have suggested that 'back-pressure' is the cause of reversal, without specifying any particular structures as being responsible, the idea being that the heart is in a complex circuit and cannot compete against the resistance which is gradually built up, and is therefore compelled to reverse.

La Hille (1880) presented a more detailed theory of back pressure, based on experiments with salps and ascidians. The essence of the theory is that the capacity of the dorsal vessels and 'lacunae' are less than the ventral, so that a congestion builds up in the ventral vessels after the heart has pumped blood in a given direction for a certain time, the heart reversing to avoid the back pressure arising from this congestion. The absence of valves of course renders this possible. A number of experiments are quoted, for example, a rapidly beating heart reversed more frequently than one working slowly because the pressure in any given direction was built up more quickly. When the creature has suffered loss of blood there are less frequent reversals, as there is less blood to congest the narrow blood channels, and build up back pressure. If *Phallusia* is treated with dilute nicotine solutions in sea water the heart beats more rapidly and the contraction of the animal, brought about by the nicotine, constricts the blood vessels, back pressure being rapidly built up and reversals becoming more frequent. Schultze (1901) and Nicolai (1908) have put forward similar alternative theories, based on the fact that during a series of contractions, the end of the heart *opposite* to the one initiating contractions shows anti-peristaltic quivers which, when the heart pauses, take over the initiation of contraction in the opposite direction.

A modified form of the back pressure theory has been put forward by von Skramlik (1930), in which reversal is associated with the periodic contraction of the whole animal, bringing about a reduction of the capacity of the blood vessels by constriction, thus facilitating a build-up of back pressure and subsequent reversal. It should also be mentioned that according to Enriques (1904) there is no true circulation in ascidians, but merely an oscillation to and fro.

THEORY

We consider that a satisfactory explanation of the reversal of the heart beat must consider the problem in two parts: first, why the heart stops after beating for some time in a particular direction, and secondly why it starts again in the reverse direction instead of merely remaining out of action until the pressure restraining it has leaked away. The theory presented below is mainly concerned with the first part of the problem.

It is demonstrated that after the heart has been beating for a certain length of time in a given direction, a back pressure is created against which the heart cannot compete and thus stops beating. The evidence for this is based on a quantitative comparison between the facts observed in living ascidians, and the theoretical conclusions arrived at when the creature is considered as a purely hydrodynamical system.

The experiments to be described only lead to speculative ideas about the second part of the problem, which, however interesting they may be, require investigation by special techniques before definite conclusions can be formed.

In order to treat the problem of the heart reversal theoretically, it is obviously necessary to make a number of simplifying assumptions.

We first of all replace the known blood vascular system of *Ascidiella aspersa* by an 'equivalent circuit' to which we can apply physical principles. The circuit immediately suggested is shown in Fig. 1. In this, the network of blood capillaries is replaced by a single tube, which offers the same hydrodynamical resistance to the flow of blood. It can be shown that for a tube of varying cross-section, the quantity of liquid passing per unit time under a pressure head is to a first approximation proportional to the pressure head provided that the inlet and outlet cross-sections are the same. This is true, of course, for a uniform bore tube as is shown by the well-known Poiseuille's formula, and as any capillary network may be built up entirely of tubes of varying cross-section arranged in 'series' or 'parallel', it may be replaced by a uniform bore tube for the purpose of the flow/pressure relation, which is all we need in this treatment. This argument also applies to the lacunar system. Now the simplified capillaries, dorsal branchial vessel and the lacunae, themselves constitute a tube of varying cross-section, and, as the branchial vessel plays no part in our theory, may be replaced by a simple tube, lettered C in Fig. 2. The ventral branchial and cardio-visceral vessels are replaced by constant wide-bore elastic tubes, lettered *A* and *B* respectively.

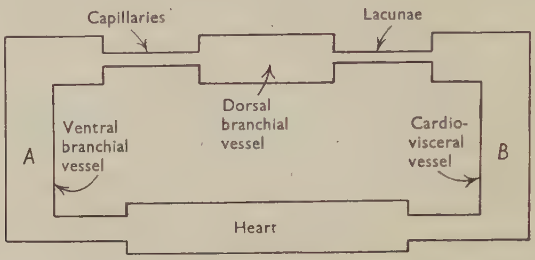

Fig. 1.

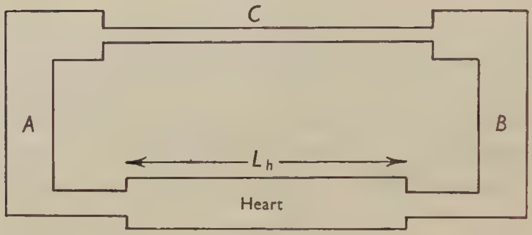

Fig. 2.

As the heart beats, shall we say from left to right, blood is withdrawn from *A* causing it to collapse, and is forced into *B*, causing it to expand. As *A* and *B* are of wide bore, the pressure in these vessels will very quickly become uniform. A pressure head will, however, exist across *C* and blood will flow through *C* from *B* to *A*. The expansion of *C* is neglected in comparison with that of *A* and *B*.

The action of the heart is regarded in the equivalent circuit as a constriction of the form shown in Fig. 3 passing along the heart. We have a cylindrical tube with

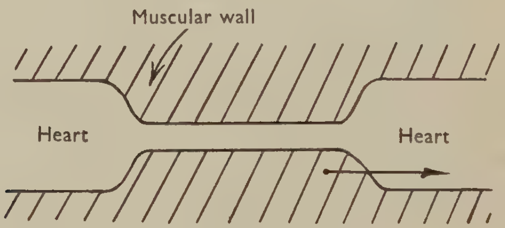

Fig. 3.

a piston having a hole bored through it moving from left to right (say). Thus the pressure head existing across *C* will also exist across the piston, and blood will therefore leak from *B* to *A* through the hole as well as through *C*.

In the living animal it is observed that when the heart constriction reaches the end of its travel, the constriction disappears, another appearing simultaneously at the opposite end of the heart. If the heart beats *n* times, this is depicted in the equivalent circuit as the piston moving continuously down a tube of length n times the actual length of the heart. This is shown in Fig. 4.

Let us denote the hydrostatic blood pressure of the system by p_0 when the heart is not beating. After n beats from left to right, the pressures will be p_1 in A and p_2 in B, where $p_1 < p_0 < p_2$, not taking into account the leakage through C and the piston leak.

Let $p_1 = p_0 + \Delta p_1$ and $p_2 = p_0 + \Delta p_2$ (Δp_1 is thus negative).

After n beats in the reverse direction, i.e. right to left, the pressures would be $p_0 - \Delta p_1$ and $p_0 - \Delta p_2$ in A and B respectively. This assumes that at the beginning of a sequence of beats in either direction the pressure is p_0, i.e. the excess pressure has leaked away during the rest period. It may actually be observed that during the rest period there is a reflux of blood into the heart from the vessel into which the blood was being pumped (Van Hasselt, 1824; Ritter, 1893). Thus we see that the pressure heads causing the flow through C and the 'heart piston leak' would be the same after equal numbers of beats in either direction. If the n beats occur in the same time for beats in either direction, we should therefore expect that the animal would behave identically with the heart beating in either direction, in spite of the fact that the blood vascular system is asymmetrical. This we shall see is borne out in practice.

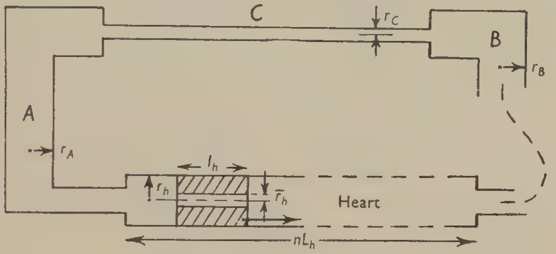

Fig. 4.

It is now assumed that blood is pumped into B (say) faster than it can escape through C and the 'heart-leak'. The fact that a puncture of the blood vessel B will give a spurt of blood of obviously considerable pressure is sufficient evidence for this, because otherwise the pressure would only be due to the *circulation* and this would obviously be very small.

If the heart beats n times in t sec. before the stoppage and subsequent 'rest period', then in the equivalent circuit, the linear velocity of the piston is given by

$$v_h = nL_h/t, \qquad (1)$$

where L_h is the length of the heart. This assumes that the heart always beats with the same velocity. In practice, the heart 'slows up' considerably for a few beats just before the rest period, but the error introduced by this in several tens of beats will be very small.

We now hypothesize that a pressure is built up by the action of the heart until the heart muscles are unable to act against it. The heart labours for a short time and then stops. We make at this stage the definite assumption that it is always at the *same* back pressure that the heart stops. We shall use the term 'back pressure' to mean the resistance to the heart due to excess pressure on one side of it, together with the diminished pressure on the other, i.e. the pressure head across the heart.

Consider the heart to be beating from left to right as shown in Fig. 4. Let the initial conditions before the heart starts beating be that the pressure in the vascular system is everywhere p_0.

After a short time δt the pressures will be:

$$\left.\begin{aligned} p_0+\delta p_1 &= p_0+\left(\frac{dp_1}{dt}\right)\delta t \quad \text{in } A, \\ p_0+\delta p_2 &= p_0+\left(\frac{dp_2}{dt}\right)\delta t \quad \text{in } B. \end{aligned}\right\} \tag{2}$$

We require expressions for the increase in volume of A and B due to these pressure changes: let them be δv_A and δv_B respectively. It may be shown* that a thick-walled elastic tube of length l, and internal and external radii a and b respectively, will have its internal volume increased by the following amount if the pressures inside and outside are raised from zero to p_1 and p_0 respectively:

$$\pi a^2 l\left\{\frac{p_1a^2-p_0b^2}{(b^2-a^2)}\frac{1}{k}+\frac{b^2(p_1-p_0)}{(b^2-a^2)}\frac{1}{m}\right\}, \tag{3}$$

where k = bulk modulus of elasticity of tube wall, and m = rigidity modulus of elasticity of tube wall.

If $p_0=p_1$, i.e. equal pressures inside and outside, then the increase will be, by substitution in equation (3),

$$-\frac{\pi a^2 l}{k}p_0. \tag{4}$$

Thus the change in internal volume Δv_1 for an increase of pressure from p_0 to p_1 inside the tube, the outside pressure being p_0, is

$$\Delta v_1=\pi a^2 l\left\{\frac{p_1a^2-p_0b^2}{(b^2-a^2)}\frac{1}{k}+\frac{b^2}{(b^2-a^2)}\frac{p_1-p_0}{m}\right\}+\frac{\pi a^2 l}{k}p_0,$$

which reduces to

$$\Delta v_1=\pi a^2 l\left\{\frac{1}{k}\left(\frac{a^2}{b^2-a^2}\right)+\frac{1}{m}\left(\frac{b^2}{b^2-a^2}\right)\right\}(p_1-p_0). \tag{5a}$$

Similarly, for a change in internal pressure from p_0 to p_2 inside the tube, the outside pressure being p_0, the change in internal volume Δv_2 is given by

$$\Delta v_2=\pi a^2 l\left\{\frac{1}{k}\left(\frac{a^2}{b^2-a^2}\right)+\frac{1}{m}\left(\frac{b^2}{b^2-a^2}\right)\right\}(p_2-p_0). \tag{5b}$$

Thus the change in volume for an increase in internal pressure from any value to any other differing by δp will be proportional to δp whatever the external pressure. That is

$$\delta v=c\delta p,$$

where

$$c=\pi a^2 l\left\{\frac{1}{k}\left(\frac{a^2}{b^2-a^2}\right)+\frac{1}{m}\left(\frac{b^2}{b^2-a^2}\right)\right\}.$$

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[*] See, for example, *Mathematical Theory of Elasticity*, by A. E. H. Love (4th ed. 1927, p. 145).

Thus we may write $\delta v_A = c_A \delta p_1; \quad \delta v_B = c_B \delta p_2;$

or
$$\delta v_A = c_A \frac{dp_1}{dt} \delta t; \quad \delta v_B = c_B \frac{dp_2}{dt} \delta t.$$

In the limit these become
$$\frac{dv_A}{dt} = c_A \frac{dp_1}{dt}; \quad \frac{dv_B}{dt} = c_B \frac{dp_2}{dt}. \tag{6}$$

$\frac{dv_B}{dt}$ is the rate of increase of the volume of B and is equal to the rate of pumping by the piston (imagined without the leak) *minus* the rate of volumetric leakage through the heart leak and through C. That is
$$\frac{dv_B}{dt} = \pi r_h^2 v_h - Q_c - Q_h, \tag{7a}$$

where r_h = radius of the heart, Q_c = leakage in c.c./sec. through C, Q_h = leakage in c.c./sec. through the heart leak. Similarly, we have for A
$$\frac{dv_A}{dt} = -\pi r_h^2 v_h + Q_c + Q_h. \tag{7b}$$

In passing we note that
$$\frac{dv_A}{dt} = -\frac{dv_B}{dt}.$$

Combining equations (6) and (7) we have
$$\left.\begin{aligned} c_A \frac{dp_1}{dt} &= -\pi r_h^2 v_h + Q_c + Q_h, \\ \text{and} \qquad c_B \frac{dp_2}{dt} &= \pi r_h^2 v_h - Q_c - Q_h. \end{aligned}\right\} \tag{8}$$

The rate of change of the pressure head across the piston is
$$\frac{dp}{dt} = \frac{dp_2}{dt} - \frac{dp_1}{dt},$$

That is, the rate of increase of the pressure in B *minus* the rate of increase of pressure in A. The latter is of course negative numerically. Thus:
$$\left(\frac{c_A c_B}{c_A + c_B}\right) \frac{dp}{dt} = \pi r_h^2 v_h - Q_c - Q_h. \tag{9}$$

Applying the well-known Poiseuille's formula, we have immediately
$$Q_c = \frac{p \pi r_c^4}{8 \eta l_c} \quad \text{and} \quad Q_h = \frac{p \pi \bar{r}_h^4}{8 \eta l_h}, \tag{10}$$

where $p = \int \frac{dp}{dt} dt$ and is thus the pressure head, r_c = radius of C, l_c = length of C, $\bar{r}_h$ = radius of heart piston leak, l_h = length of heart piston leak, η = viscosity of the blood.

Substitution from equations (10) in equation (9) yields
$$\left(\frac{c_A c_B}{c_A + c_B}\right) \frac{dp}{dt} = \pi r_h^2 v_h - p\left(\frac{\pi r_c^4}{8 \eta l_c} + \frac{\pi \bar{r}_h^4}{8 \eta l_h}\right). \tag{11}$$

This is a differential equation of the form

$$a\frac{dp}{dt}+bp-c=0, \tag{12}$$

the solution giving a relation between the back pressure p and the time t for given values of the constants a, b and c. The solution is

$$p=\frac{c}{b}(1-e^{-bt/a}). \tag{13}$$

Substituting from equation (1) for v_h in c, this becomes

$$p=\frac{\pi r_h^2}{b}(1-e^{-bt/a})\frac{nL_h}{t}. \tag{14}$$

Thus the time taken for the back pressure to reach the critical value p' at which the heart stops beating is given by T in the equation

$$p'=\frac{\pi r_h^2}{b}(1-e^{-bT/a})\frac{nL_h}{T}, \tag{15}$$

which may thus be regarded as an equation relating the time of beating of the heart in one direction, T, before reversal, to the number of beats in that direction, n. It is convenient, however, to keep as variables the quantities T and n/T, and as in the experiments it is necessary to vary the temperature of the animal, the viscosity must appear explicitly in the equation.

Substituting the values of a, b and c from equations (11) and (12) in equation (15) we obtain eventually an equation of the form

$$\eta\frac{n}{T}=\alpha\left(\frac{1}{1-e^{-\beta T/\eta}}\right), \tag{16}$$

where α and β are both constants and depend only on the dimensions of the blood vessels, their elasticities and the critical pressure p'.

The experiments were devised to attempt to establish the validity of this equation, the observational variables being taken as $\eta(n/T)$ and T/η.

The speeding up of the heart beat of ascidians with rise in temperature is very pronounced and consequently affords a convenient method of varying $\eta(n/T)$, as this is proportional to the velocity of the heart beat. T can also be observed and the results used to establish the functional relation that has been derived (equation 16).

In order to compute the values of $\eta(n/T)$ and T/η it is necessary to know the variation of η with temperature. The experimental methods for determining this variation and also for the verification of equation (16) are described below.

THE EXPERIMENTS

The results given in this paper were obtained from observations on *Ascidiella aspersa* (O. F. Mueller). The test of *A. aspersa* is clear and firm, but the crinkled surface makes direct observation of the heart impossible except in occasional specimens. A thin layer was shaved off the test just above the heart, the beating of which could then clearly be seen through the test.

In earlier experiments, a 'window' was cut in the test but the exposure of the pericardium made the animal behave abnormally. Hecht (1918), working with *A. atra*, found a similar effect. The test was shaved immediately after collection and the animal was allowed to recover in gently running sea water for 24 hr. before being used in an experiment. The *Ascidiella* were collected from various positions in Plymouth Sound: Asia Shoal and Millbay Dock.

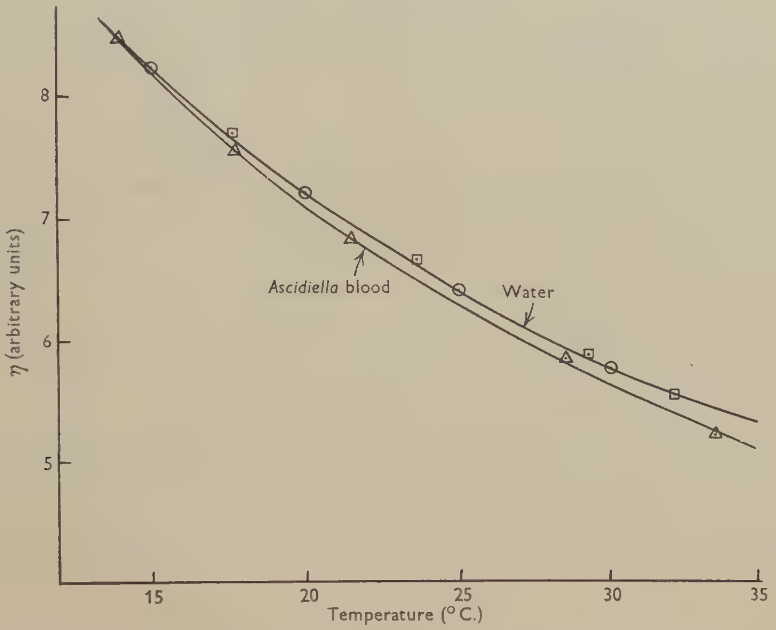

Fig. 5. Variation of viscosity with temperature. ⊙, water: accepted values (Kaye and Laby, *Chemical and Physical Constants*); ⊡, water: experimental values; △, *Ascidiella* blood: experimental values.

EXPERIMENTS TO DETERMINE THE VISCOSITY-TEMPERATURE RELATION FOR *ASCIDIELLA* BLOOD

The determination of the viscosity of *Ascidiella* blood presented considerable difficulties owing to the extremely small quantity available for experiment.

A method was devised involving the rate of fall of a small 'pellet' of the blood in a capillary tube. The experimental and theoretical details of the method are not relevant to this paper, but in view of possible applications of the method, one of us (C.A.H.) hopes to publish them elsewhere.

In the present theory the absolute magnitude of the viscosity of *Ascidiella* blood is not required, as the constant of proportionality involved can be incorporated in the constants α and β.

The results obtained for water, *Ascidiella* blood and the accepted values for water are shown in Fig. 5 where the vertical axis has been chosen arbitrarily. It will be seen that the viscosity of *Ascidiella* blood varies with temperature in approximately the same way as water over the range we have used.

EXPERIMENTS TO VERIFY THE RELATION $\left(\eta \frac{n}{T}\right)=\alpha\left(\frac{1}{1-e^{-\beta T/\eta}}\right)$

The experiments were performed with the animal wedged by pins on a weighted square of cork in a finger bowl of sea water. For experiments below room temperature, the bowl was immersed in a large Petri dish of ice and water, and by varying the amount of ice it was possible to bring the temperature down to the desired values. For temperatures above room temperature, the bowl was placed in a copper 'warm filter funnel' which was heated by a spirit lamp. The temperature of the bowl was regulated by controlling a flow of cold water around the bowl inside the funnel.

The range of temperatures used was 4–30° C. After exposure to extremes of temperature, the animal was given a change of sea water and allowed to settle down for at least 10 min. In view of the intimate contact of the water with the blood vessels the animal must follow temperature changes very closely. Temperatures were recorded to the nearest $\frac{1}{10}$° C. at the beginning and end of each sequence of beats, the mean value being used to calculate the blood viscosity.

The beats were recorded on two post-office type call counters and the times were kept by stop-watches.

Sets of readings for temperatures above and below room temperature were alternated, the range being gradually widened so that should the animal die in the course of the experiment, the results would not be completely vitiated. This was found to be unnecessary with *Ascidiella aspersa*, as even 10 days after being exposed to the temperature extremes mentioned, the specimens were found to be perfectly healthy.

RESULTS

Figs. 6–10 show the variation of $\eta(n/T)$ against T/η in graphical form for five specimens of *A. aspersa*. The full lines represent relations of the form given in equation (16), the constants α and β being calculated by solution of equation (16) for two observed pairs of values of the variables. A certain amount of choice is thus available.

It will be seen that as the velocity of the heart increases, the reversals take place more frequently, the observed results agreeing with the theoretical relation, we suggest within the normal biological limits.

The 'scatter' from the theoretical curve is, of course, not due to experimental error: it is a very real deviation from the theoretical, and we consider the interpretation of the scatter to be important. In developing the theory we made several simplifying assumptions: assumptions which would be modified in the living animal by the numerous external and internal variations which are perhaps beyond the scope of theory, e.g. growth, irritability, physical movement, fluid interchange between the blood and the sea water, etc. It is only the *trend* of the observations which can be considered. It is this that we submit is in agreement with the theoretical relation and shows that the actual stopping of the heart is due to the back pressure that has been built up.

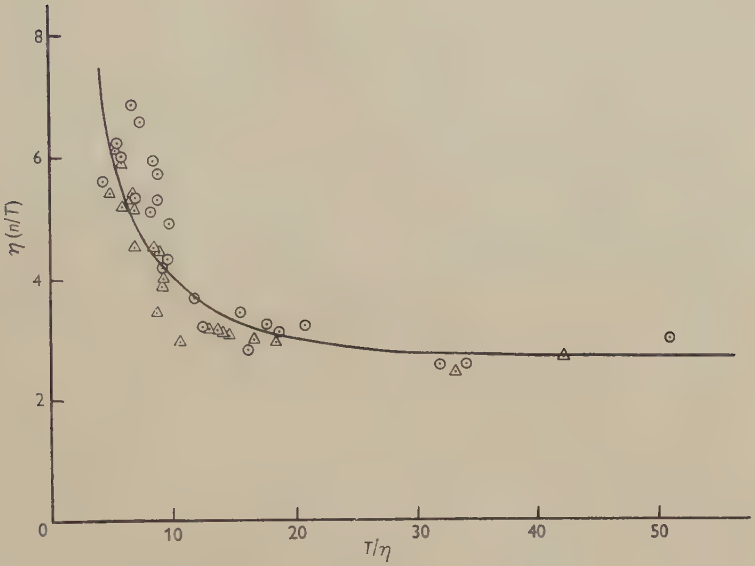

Fig. 6. *Ascidiella aspersa* I. $\alpha = 2{\cdot}66$; $\beta = 0{\cdot}11$. ⊙, abvisceral beat; △, advisceral beat.

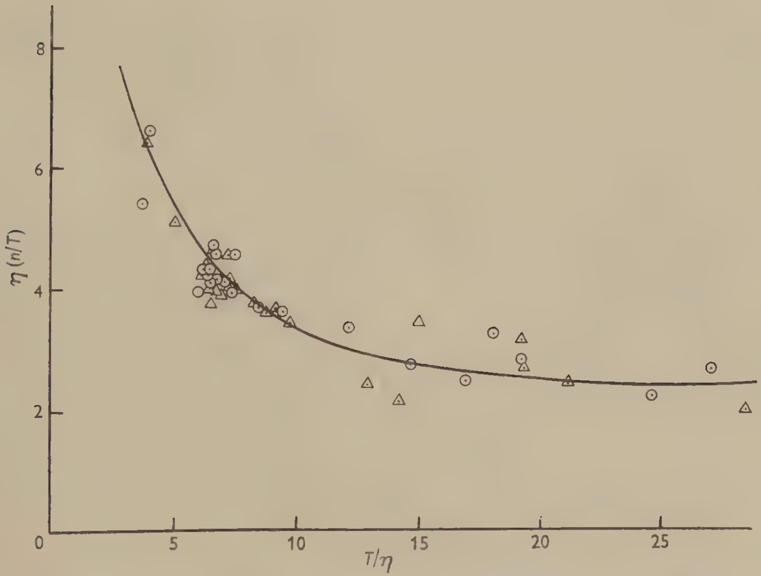

Fig. 7. *Ascidiella aspersa* II. $\alpha = 2{\cdot}07$; $\beta = 0{\cdot}096$. ⊙, abvisceral beat; △, advisceral beat.

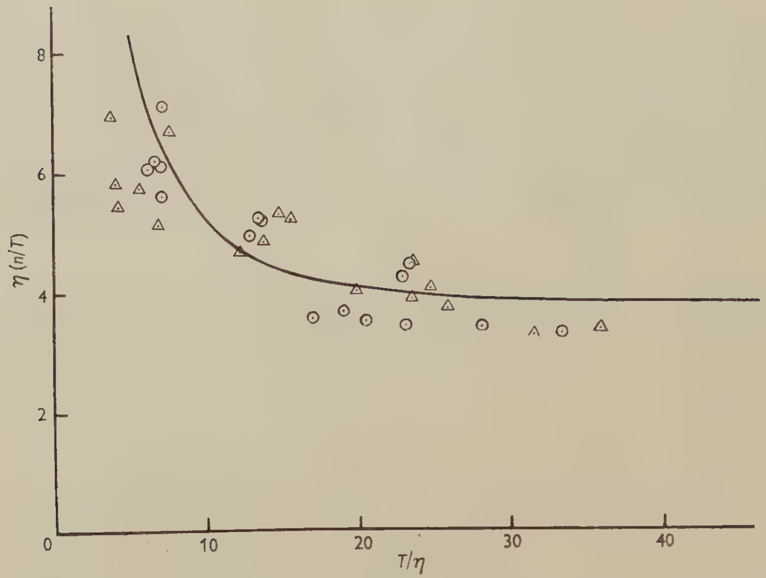

Fig. 8. *Ascidiella aspersa* III. $\alpha = 3{\cdot}90$; $\beta = 0{\cdot}139$. ⊙, abvisceral beat; △, advisceral beat.

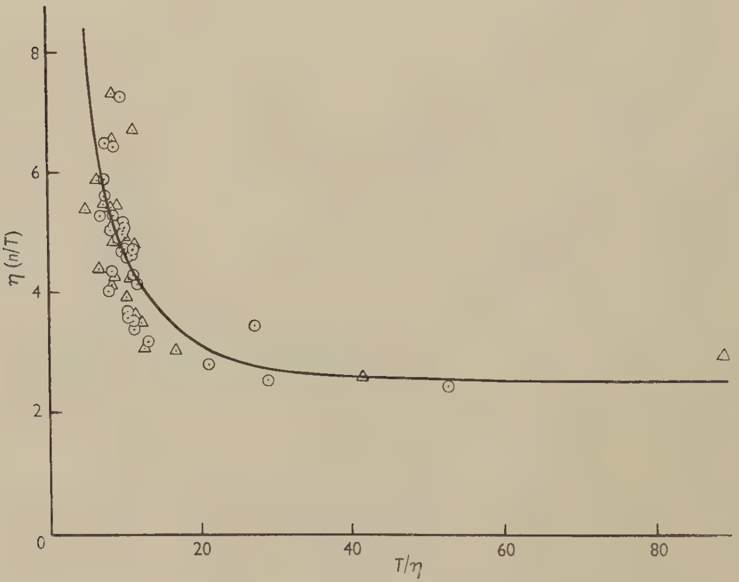

Fig. 9. *Ascidiella aspersa* IV. $\alpha = 2{\cdot}33$; $\beta = 0{\cdot}076$. ⊙, abvisceral beat; △, advisceral beat.

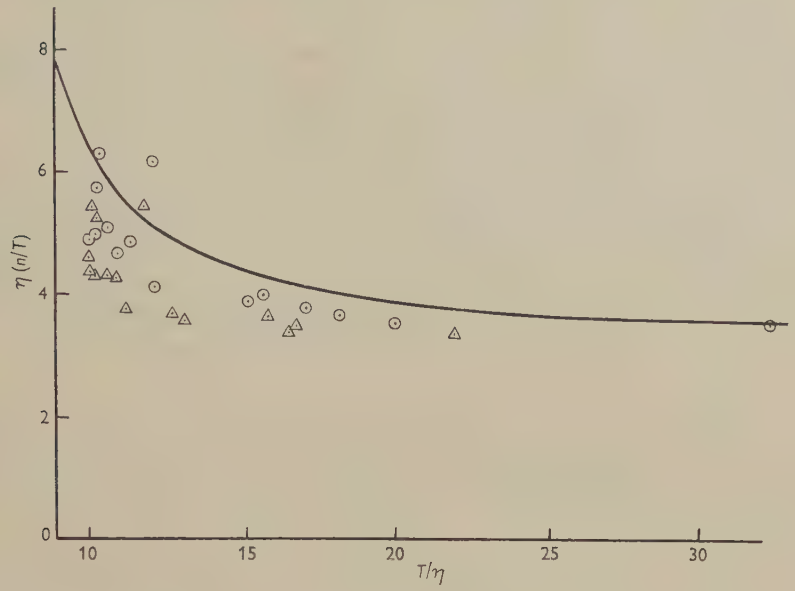

Fig. 10. *Ascidiella aspersa* V. $\alpha=3\cdot0$; $\beta=0\cdot70$. ⊙, abvisceral beat; △, advisceral beat.

DISCUSSION

The second part of the problem: why the heart reverses after pausing, is almost certainly bound up in some way with the capacity of each end of the heart to initiate contractions. In a heart completely removed from the animal, we have observed the phenomenon of the heart contracting from both ends simultaneously, waves passing from each end to the middle, and occasional antiperistaltic quivers may be observed in the intact animal.

Some observers have reported reversal in the isolated heart. We have not observed this phenomenon, but there seems to be no reason to doubt that it may occur. We should not, however, expect regular reversals. This phenomenon is in no way incompatible with the back-pressure theory as has been suggested (Hecht, 1918; Bancroft & Esterley, 1903; Krukenberg, 1880), because each end of the heart is capable of initiating contractions and any random disturbance might well make one set of contractions preponderate in the absence of any external 'guiding' forces such as are met with in the build-up and consequent action of the back pressure.

A further criticism often quoted against the back-pressure theory is the observation that the heart of *Ascidia atra* ceases to reverse above 35° C. (Hecht, 1918), and therefore back pressure is not built up, otherwise the heart could not beat continuously in one direction. It should be stressed that 35° C. is so far above the optimum temperature that the animal is abnormal, and 'one way' beating is a typical symptom of a damaged animal. Under such circumstances it is readily observed that the actual constriction of the heart is much less than in the normal animal. Thus the work done by the heart is much reduced and the 'heart leak' is much increased. Equilibrium may be set up and the pressure may never reach the critical value p'. In equation (9) the pressure is increasing with time if $\pi r_h^2 v_h - Q_c - Q_h$ is positive. In the damaged animal referred to above Q_h is much increased and may result in the above expression becoming zero (it cannot become negative), thus $dp/dt = 0$ or $p =$ constant which can be less than p'.

Finally, objections have been raised on the grounds that the back-pressure theory reduces the heart to a purely mechanical device, whereas it is clearly a living system (Hecht, 1918). Obviously, the fact that the heart is a contractile tube of living cells does not mean that the contained fluid is not subject to ordinary physical laws. The slowing up of the heart beat just before reversal is very suggestive of a back-pressure having been built up. Many observers have recorded this slowing up (Alder & Hancock, 1905; Hancock, 1868; Van Hasselt, 1824; Herdman, 1924; Lister, 1834; Wagner, 1866).

The conclusions put forward by von Skramlik (1930) and Enriques (1904) are not borne out by our observations, nor apparently by those of any other workers.

The reversal of the tunicate heart is a characteristic and deep-rooted feature, and is seen in the heart of the larva from the moment it starts to contract (Berril, 1935, 1947), while in regressing colonies of *Botryllus* and *Botrylloides* the heart continues to beat long after the other tissues have broken down (Pizon, 1899, 1900).

Although the reversal of the heart beat of tunicates is usually considered to be unique, it is of some interest to note that there are other recorded cases of reversing systems. Examples are the fluids of embryos of *Limax* and *Planorbis* (van Beneden & Windischmann, 1841; Schmidt, 1851) and the blood systems of *Phoronis* (Bethe 1927) and the parasitic copepod *Lepeoptheirus pectoralis* O. F. Müller (Scott, 1901). Gerould (1929*a*, *b*, 1930, 1933) records numerous cases of the reversal of the heart beat in insects, while Tirelli (1936) produced evidence that in the moth *Saturnia*, back pressure might well be the factor causing the heart to pause. Finally, Gowanlach (1923), working with the teleost fish *Macropodus viridi auratus* Lacepede, produced by chemical treatment a tubular heart in the embryo which reversed regularly, similar to a tunicate heart.

One concludes that the reversal of the tunicate heart may not be as unique as is usually supposed and that in any system where a contractile tube, devoid of efficient valves, has to compress a fluid and force it round a circuit, back pressure is liable to build up and to result in reversal.

SUMMARY

1. The reversal of the tunicate heart involves two problems: first, why it stops beating in a particular direction; and secondly, why having stopped it should then reverse.

2. Two types of theory have been put forward, one based on the idea that a 'back pressure' is gradually built up which compels the heart to stop, and the other depending on opposing centres of contraction at each end of the heart taking control alternately.

3. The present paper provides quantitative evidence, based on *Ascidiella aspersa* (O. F. Mueller), that a back pressure is built up. The faster the heart beats the more

frequently reversals occur, a back pressure building up more rapidly when the heart beats quickly.

4. Various criticisms of the back-pressure theory are considered and discussed.
5. Examples are given of other closed contractile systems containing fluid which show reversal, the tunicate heart being by no means unique.

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OF THE MOVEMENT OF WORMS

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(With Three Text-figures)

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I. INTRODUCTION

There is little published work dealing with the movement of worm-like animals, apart from descriptive papers such as that of Foxon (1936), and analyses of the application of muscular contraction to the substratum such as are found in the papers of Gray (1939) and Gray & Lissmann (1938*a*, *b*). The possession of the right volume of coelomic fluid was shown by Chapman & Newell (1947) to be important in the burrowing of the lugworm, and the function of the body fluid in the extrusion of the proboscis of *Nephthys* and in the movements of *Calliactis* has been demonstrated (Chapman, 1949 and 1950). There appears, however, to be no satisfactory account of the interaction of the antagonistic muscles which is made possible by the presence of the body fluid, although von Buddenbrock (1937) mentions the antagonism between the longitudinal and circular muscles and describes their nervous co-ordination.

In the present paper an attempt is made to examine theoretically the working of antagonistic longitudinal and circular muscles in cylindrical fluid-filled animals. In the movement of these creatures there are features upon which it is necessary to lay stress, and although they may not all be self-evident they may, for convenience, be stated concisely until a more rigorous proof can be given.

(*a*) The longitudinal and circular muscles are antagonists which depend for their antagonism upon the presence within the animal of a fluid-filled cavity of fixed volume. (The fluid-filled cavity varies in size in different animals and although, in many, the volume of coelomic fluid is small, nevertheless that which is contained within the body wall may be regarded as fluid and incompressible for the purposes under review.)

(*b*) The maximum thrust which the musculature of an animal can exert is measured by the internal hydrostatic pressure and the area of application. The maximum pressure can be increased above the internal hydrostatic pressure by the application of the principle of the wedge.

(*c*) The maximum thrust which the animal can exert is commensurate with the friction forces available to resist it.

(*d*) The absolute change in length of the animal which can be brought about by a given percentage contraction of the circular muscles depends on the shape of the animal, i.e. upon the relation of length to diameter.

It is necessary, then, to put forward some justification for the previous statements, and to examine their bearing on the shapes, sizes and muscular construction of soft-bodied invertebrates.

II. THE ANTAGONISM OF CIRCULAR AND LONGITUDINAL MUSCLES

It has been understood since the time of Leonardo da Vinci that a muscle 'uses its power along the line of its length'. In general terms this power is exerted during contraction, and a muscle is unable to exert its power again until it is restored to its resting length by an external agent. Spontaneous elongation of the muscles of the starfish podium has been reported by Paine (1929), but Smith (1946) has clearly refuted the suggestion and has collected considerable experimental and histological evidence to show that it does not occur, the elongation of the isolated tube foot being due to the unfolding of the crumpled collagen fibres which can exert sufficient force slowly to stretch the muscle.

To restore its 'useful' (relaxed) state a muscle may have in opposition to it either the force of another muscle, an elastic force or possibly a ciliary pressure. It may be noted that the force developed by the restoration system need not be as great as that developed by the effector. If the muscle works against an elastic restoring agent the work which it can perform externally is diminished by the work which is required to deform the elastic body.

The deformation of an elastic substance and the use of ciliary pressure cannot be regarded as important methods of restoration in the animal kingdom as a whole, although elasticity may play a part in the functioning of the muscular system of nematodes and ciliary pressure in that of coelenterates. Whilst it is true that ciliary pressures are of the order of mm. of water only (Pantin, private communication), it is also true that very small forces suffice during life to extend the body wall of certain actinians (see, for example, Jordan, 1935).

If a muscle works against an antagonistic muscle the work which it can perform externally is diminished only by the work necessary passively to extend the antagonist through the transmission system by which its force is applied. In many of the lower invertebrates this takes the form of a hydrostatic skeleton consisting of a fixed volume of liquid or tissue which is in a practically fluid state during life. The liquid may be the contents of the enteron, the coelom or the haemocoel, but one of its functions in all instances is that of a pressure-transmission system which causes the effects of contraction of any one muscle automatically to be felt by all the remaining muscles in the body wall. The contraction of a muscle in the body wall running parallel to the surface would cause a diminution in the volume enclosed by the body wall if the fluid were compressible. Since, however, it is incompressible the contraction of any one muscle must bring about an increased internal pressure and an extension of the remaining muscles.

Any fluid-muscle system which consists of a fluid surrounded by a muscular wall has inherently in its construction its muscular restoring agent, provided that muscle fibres of the wall run in longitudinal and circular directions, or that the skeletal materials of the wall contain an element of elasticity in their physical properties. This can be seen by considering a hypothetical animal whose body wall consists of circular fibres only and lacks any skeletal elasticity. Contraction of the muscles at one end could bring about a thinning of that end, and *either* an elongation or a thickening at the other (see Fig. 1). If this thickening were to take place, all that would be necessary to restore the original condition would be a contraction of the dilated end. But, on the other hand, if an elongation were to occur, there would be no way of getting back to the original condition since contraction of the muscles in the elongated region would serve only to relax the muscles in the original contracting end, and so to thicken it without shortening the body as a whole (see Fig. 1). It is interesting to note here that, where only a single muscular coat occurs in a hollow organ such as a starfish podium, there is a strong inelastic sheath of collagen fibres which prevents increase in diameter of the foot on contraction of its muscles. When this occurs the contained fluid is driven into the ampulla, which together with the tube foot, acts as a closed system.

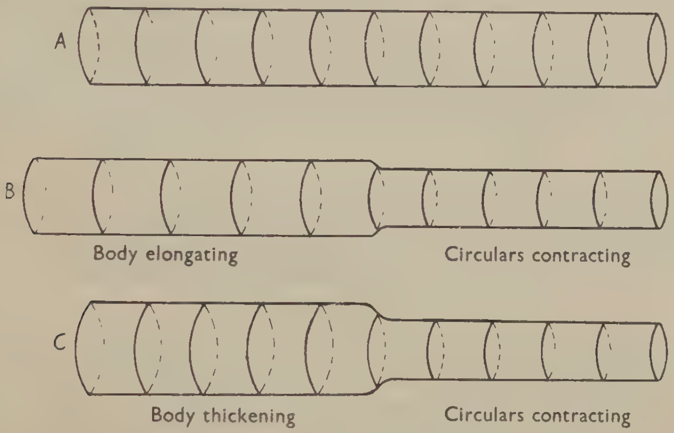

Fig. 1. Diagram to illustrate the action of a hypothetical animal with circular muscles only. In *A* all the muscles are relaxed. In *B* the muscles of the right-hand end have contracted but the length of this end has remained the same. If this takes place it is impossible for the original length of the right-hand muscles to be restored by the contraction of the left-hand muscles. In *C* an alternative arrangement is shown. The muscles of the right-hand end have contracted, but the muscles of the left-hand end have relaxed. There has been no change in length. By the contraction of the left-hand muscles the right-hand muscles could be restored to their original length.

In most soft-bodied invertebrates the musculature of which the body wall is composed is arranged according to a uniform plan in which the outer layer consists of fibres running in a circular manner, whilst the inner layer of fibres runs along the length of the animal. This arrangement enables the muscles to act antagonistically to one another without the necessity for the body wall to possess any skeletal elastic component, provided that the nervous system arranges for the muscles to be suitably stimulated. If the action of an animal in which there are circular and longitudinal muscles be compared with that of the hypothetical organism consisting of circular muscles only, it can be seen that the contraction of the circular muscles at one end can bring about four different muscular configurations which are illustrated in Fig. 2. Restoration of the original body shape from any of these states can be brought about by the *contraction* of appropriate other parts of the musculature as can be seen on inspection of the figure.

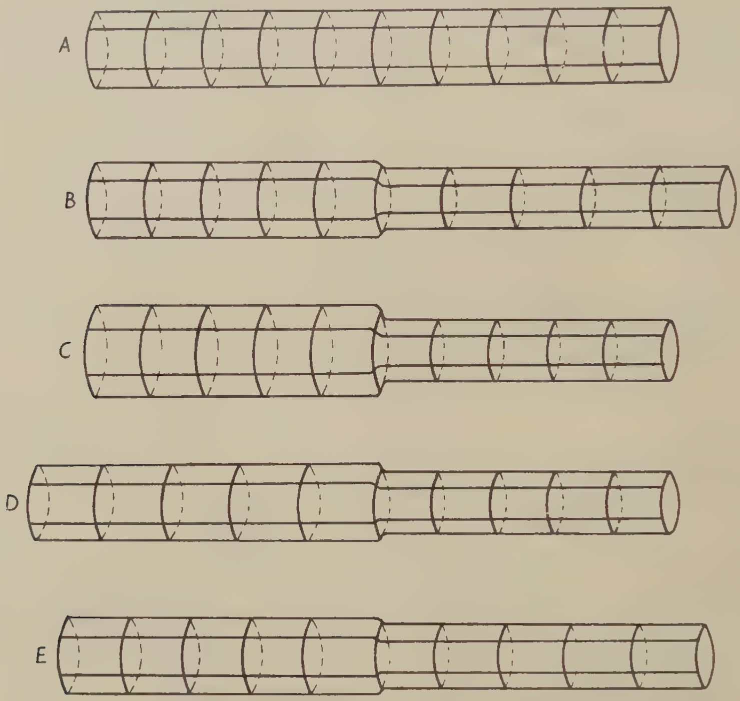

Fig. 2. Diagram to illustrate four possible results of the contraction of circular muscles at one end of a cylindrical animal. In *A* the muscles are all relaxed. In *B* the circular muscles of the right-hand end have contracted and this end has elongated. The left-hand end has remained unaltered. In *C* the length of the right-hand end has remained the same but the diameter of the left-hand end has increased. In *D* the length of the right-hand end has also remained the same. The length of the left-hand end has increased but not its diameter. In *E* the length of both ends has increased but their diameters have remained the same as in *B* and *D*.

That the ordering of the muscle fibres in a longitudinal and circular manner is the only arrangement which makes possible the movements of soft-bodied invertebrates can be seen by considering the results of the arrangement of the fibres at an angle of 45° to the long axis in such a way that they are arranged in two spirals of opposite sense. The effect of the contraction of one spiral on the dimensions of the body would be the same as that of the other so that neither could be said to be in opposition to the other nor do they constitute a mutually restorative system. On the other hand, the arrangement of inextensible skeletal fibres in this manner allows change of dimensions of the animal at fixed volume without stretching of the skeletal material.

The antagonism of relaxed muscles to those which are contracting depends on the presence of a body fluid which in its turn necessitates a non-leaking body wall. In those animals which have been examined this has been found to occur. In *Arenicola* the nephridiopores are sphinctered apertures capable of resisting a pressure greatly in excess of that normally produced by contraction of the body-wall muscles. Loss of body fluid causes loss of burrowing efficiency, which is prevented on mutilation by the marked constriction of the body wall on to the gut (Chapman & Newell, 1947). In *Calliactis* practically no water leaves the stomodaeum or the cinclides on normal contraction (Chapman, 1950). In *Lumbricus* free flow of fluid about the body is prevented, and escape of fluid on mutilation is hindered, by the presence of muscular septa; neither do the dorsal pores nor the nephridiopores leak during life (Newell, private communication).

III. HYDROSTATIC PRESSURE AND MUSCULAR TENSION

The relation between the pressure in a hollow cylinder and the stresses in its walls can be seen by considering the forces exerted on a longitudinal section passing through the axis and also the forces on a cross-section.

If 't' is the thickness of the wall, 'l' the length, 'r' the radius and 'f_c' and 'f_l' the circumferential and longitudinal stresses respectively, the total push due to the pressure on one half of the longitudinal section equals $p2rl$. This must be balanced by the circumferential stress in the walls, viz. f_c2tl. Therefore

$$p2rl=f_c2tl, \quad p=\frac{f_c2tl}{2rl}=\frac{f_ct}{r}.$$

Similarly, for the cross-section the total pressure on the ends must be balanced by the longitudinal stresses in the walls, viz. $p\pi r^2=2\pi rtf_l$. Therefore

$$p=\frac{2\pi rtf_l}{\pi r^2}=\frac{f_l2t}{r}.$$

Considering now the tensions in the longitudinal and circular muscles of a worm, and the pressures which they exert on the contained fluid, T_c can be written instead of the expression f_ctl for the circumferential stress as being the total tension in the whole circular muscle layer so that

$$p2rl=2T_c.$$

Therefore
$$p=\frac{2T_c}{2rl}=\frac{T_c}{rl}.$$

Similarly, the total tension in the whole longitudinal muscle layer, T_l, can be written for the expression $2\pi rtf_l$ so that the equation becomes

$$p\pi r^2=T_l.$$

Therefore
$$p=\frac{T_l}{\pi r^2}.$$

From the expressions relating pressure with longitudinal and circumferential stresses it can be seen that

$$\frac{Tc}{rl}=p=\frac{T_l}{\pi r^2},\quad \frac{Tc}{rl}=\frac{T_l}{\pi r^2},\quad \frac{T_l}{T_c}=\frac{\pi r^2}{rl}=\frac{\pi r}{l}.$$

It follows from this expression that if the ratio of the tensions in the longitudinal and circular muscles is equal to $\pi r/l$, then the pressures being exerted on the contained fluid by the two muscle layers are equal. Whilst, therefore, there is no doubt that this equation may hold good at any instant, it is of interest to inquire if the quantities of longitudinal and circular muscle present in a typical 'worm' are such that the equation holds good when both layers are contracting maximally. This can be discovered by inspection, if it is assumed that the tension which a muscle can exert is proportional to its cross-sectional area. If A_l and A_c are the total cross-sectional areas of the longitudinal and circular muscles respectively, then

$$\frac{A_l}{A_c}=\frac{\pi r}{l}.$$

Average measurements made from *camera lucida* drawings of sections of earthworms of different sizes are set out in Table 1.

Table 1. *Dimensions of body-wall muscles of* Lumbricus

(Dimensions in mm. and sq.mm.)

	Large worm	Medium worm	Small worm
Approximate length before fixation	150	110	60
Radius	2·7	1·8	1·0
Radial thickness of circular muscles	0·1	0·05	0·04
Total cross-sectional area of circular muscles	15·0	5·5	2·4
Radial thickness of longitudinal muscles	0·3	0·3	0·2
Total cross-sectional area of longitudinal muscles	5·9	3·0	1·2
A_l/A_c (approx.)	0·4	0·5	0·5
$\pi r/1$	0·05	0·05	0·05

It can be seen from the table that in the earthworm A_l/A_c is not equal to $\pi r/l$, implying either that the tension exerted by the muscles is not proportional to their cross-sectional area or, which is much more likely, that at their maximal contraction the circular and longitudinal muscles are not balanced. From the values of $\pi r/l$ calculated from the values of 'r' and 'l' it would appear that, for equal pressure to be exerted by circular and longitudinal layers, T_c should be approximately $20\times T_l$ (or A_c should be approximately equal to $20\times A_l$) whereas, in fact, A_c is only approximately $2\times A_l$. If, then, both sets of muscles in the worm were to contract maximally, the pressure exerted by the longitudinals ($T_l/\pi r^2$) would be approximately ten times that exerted by the circulars (T_c/rl).

Since contraction of the circular muscles gives rise to a forward thrust they might have been expected to be the stronger, for they would seem to be the more important both for progression and for burrowing through the soil. However, since the animal probably burrows largely through existing crevices, the longitudinal muscles may be of greater importance since they serve to increase the diameter of the body and hence to thrust aside the soil particles. (This argument presupposes, of course, that the body is totally enclosed by the soil, because otherwise the longitudinal muscles can generate only as much pressure as the circular muscles are capable of withstanding.)

The maximum pressures in both *Arenicola* and *Lumbricus* occur during phases of contraction of the anterior circular muscles when the animals are unconfined, but in *Arenicola*, enclosed in a glass tube, values of between 80 and 90 cm. of sea water have been recorded as compared with the highest values of 50–60 cm. of sea water previously registered (Chapman & Newell, 1947). This pressure is not as great as the theoretical maximum but it shows, nevertheless, that greater pressures can be exerted by the longitudinal than by the circular muscles.

It is clear that the hydrostatic pressure in the body fluid is a measure of the pressure which can be applied to the substratum, so that the measurement of hydrostatic pressure is a convenient way of measuring the 'strength' of soft-bodied invertebrates, although the total pull which can be exerted by an animal during the contraction of its longitudinal muscles cannot be measured by the measurement of hydrostatic pressure since the transmission of the muscular tension does not require the intervention of any fluid. In this connexion it is interesting to note that the figure given by Gray & Lissmann (1938*a*) for the maximum pull which an earthworm can exert (70 g.) is in excess of the thrust which it normally exerts on extension (2–8 g.). This figure of 2–8 g. is in good agreement with the hydrostatic pressure as measured by Newell. His values have a maximum of about 30 cm. of water, i.e. 30 g./sq.cm. For a worm of diameter 0·6 cm., of cross-sectional area 0·28 sq.cm., the total thrust would therefore be about 8·5 g.

In order to compare the pressure exerted by the circular muscles with the hydrostatic pressure it is necessary to assume, as has been done before, that the tension which a muscle can exert is proportional to its cross-sectional area. If this is done, and the pull exerted by the longitudinal muscles is used as a standard, it is possible to compute the pressure which ought to be exerted by the contraction of the circular muscles. If the pull due to the longitudinal muscles of an earthworm of about 0·3 cm. radius is 70 g., the cross-sectional area of its longitudinal muscles being 0·06 sq.cm., then the tension in the circular muscles, of cross-sectional area 0·15 sq.cm., should be $\frac{70\times 0{\cdot}15}{0{\cdot}06}$ g. The pressure exerted on the contained fluid (T_c/rl) should therefore be $\frac{70\times 0{\cdot}15}{0{\cdot}06\times 0{\cdot}3\times 15}$ g./sq.cm. This value, of 38 g./sq.cm., is again in quite good agreement with the measured value of 30 cm. of water.

It is interesting to notice here that the pressure exerted by the circular muscles is not dependent upon their simultaneous contraction along the total length of the animal if the creature is capable of being divided into water-tight compartments. If, for example, a third of the length is in contraction, then the cross-sectional area of the circular muscles in this portion will be a third of the total circular muscles in the body, but so will the value of 'l' in the equation

$$\text{pressure}=\frac{T_c}{}$$

If, on the other hand, the body cavity is open and the fluid in the region undergoing contraction is in communication with the remainder, then the development of pressure in the body cavity is dependent upon the development of tension in the muscles as a whole. Examples of the open and septate coelom are seen in the lugworm and the earthworm respectively.

IV. THE APPLICATION OF THE INTERNAL PRESSURE TO THE SUBSTRATUM

Whether the thrust is applied by the extrusion of the proboscis as in *Nephthys* and *Nereis*, or by the extension of the anterior end as in *Lumbricus*, the effective application of the thrust resulting from the contraction of the body-wall muscles depends upon the ability of the animal to withstand the reaction to the thrust.

Although it may perhaps unduly extend the meaning of the term friction to include the resistance provided by parapodia and setae, nevertheless the forces exerted by these portions of the animal must be included with the friction of the body. Frictional forces have been measured for the earthworm by Gray & Lissmann (1938*a*), but no measurements of the frictional forces of the animals in their natural environment appear to have been made. Friction between an earthworm and the walls of its burrow may be considerable, since animals can easily be broken when attempts are made to pull them out. Presumably, therefore, if the longitudinal muscles of *Lumbricus* are capable of exerting a tension of 70 g. the frictional resistance which must be overcome in pulling a worm from its burrow can be at least 70 g.

It is also worth comment that the contraction of the longitudinal muscles serves to increase the diameter of the body and therefore may increase the pressure applied at right angles to the surface of the body of the worm.

If the frictional force which must be overcome for movement to occur be 'f', then the coefficient of friction is f/n, where 'n' is the normal reaction between the applied surfaces. In Gray & Lissmann's example of an earthworm moving over a glass plate the frictional resistance was 2–8 g., say 5 g., and the weight of the worm was, say, 5 g. The coefficient of friction is therefore 1. If the coefficient of friction is the same for a worm being pulled from its burrow and if $f=70$ g., then, since $70/n=1$, $n=70$, or the total reaction between the worm and its burrow is 70 g. Taking the superficial area of the worm to be 25 sq.cm. the pressure of the worm on its burrow is 70/25 g./sq.cm. This figure, of less than 3 g./sq.cm., is obviously well within the limits of the pressure occurring in the body fluid. Even if it were to be multiplied by a factor of 10, as would be necessary if only one-tenth of the superficial area of the worm were in contact with the wall of the burrow, it would imply a pressure of 30 g./sq.cm., which it is still well within the animal's power to generate.

It would appear to be fallacious to assume, as has in fact been done, that the coefficient of friction between the earthworm and a smooth plate is the same as that between a worm and its burrow, although the presence of mucus in both instances might well have the effect of lessening any differences. Some rough measurements with a dynamometer, made by dragging eight fresh *Arenicola* along the surface of drained sand, showed that each animal required a force of 10 g. weight to move it against the force of friction. The freshly dug worms which were used in this experiment weighed about 10 g. each so that the coefficient of friction between drained sand and the animal was about 1, which does not differ from the value deduced from Gray & Lissmann's observations on the earthworm. Forces involving the displacement of particles of muddy sand are not reliable quantities to measure, and not too much significance can be attached to these results.

To sum up, the figure of 30 g./sq.cm. of water necessary for an earthworm to resist a pull of 70 g. when one-tenth of its length is in its burrow is well within the maximum pressure which the worm is capable of exerting.

Newell (private communication) has pointed out that an earthworm can increase the pressure which it applies to the substratum by reducing the area in contact with the soil. If the anterior end of the animal is regarded as a truncated cone and if the internal pressure be applied to the larger end of the cone, the pressure exerted on the soil by the small end will be in the inverse ratio of the areas of the two ends. In this way, working at an internal pressure of 30 g./sq.cm., and with the area of application of the force one-twentieth that of the cross-sectional area of the worm, a force of 600 g./sq.cm. or 8 lb./sq.in. can be applied. If the total cross-sectional area of the worm is 0·25 sq.cm., then the diameter of the smaller area of application of the force would be between 1 and 2 mm. This figure certainly appears to be of the right order. It will be realized that this is a considerable pressure when compared with that of 2·5 lb./sq.in. exerted on the ground by a man standing on both feet; and it is highly likely that the tissue of the animal would be deformed by such pressure so that the area of contact would be immediately increased. It is worth noting, however, that the anterior end of an earthworm is a nearly solid muscular region which can be made rigid by contraction. It will also be seen that, since the forward thrust is expressed as a pressure, i.e. force per unit area, it implies that no increase is necessary in the frictional forces (between the animal and the ground) which resist the forward thrust.

V. CHANGES OF MUSCLE LENGTH AND BODY SHAPE

In their simplest form, taking no account of the changes in shape of the muscles themselves, the variation in length and circumference of a cylindrical animal can be regarded as those changes which take place in a cylinder of variable dimensions and fixed volume, V.

$$V = r^2 l = \text{a constant}.$$

Therefore

$$l = \frac{V}{r^2},$$

from which it can be shown that

$$\frac{dl}{l} = \frac{-2dr}{r} \quad \text{(Blaney, private communication).}$$

That is, a percentage change in length is accompanied by half that percentage change in circumference. For example, the dimensional changes in a cylinder of fixed volume of 15,700 cubic units are given in Table 2, a radius of 5 units and a length of 200 units being proportions not unlike those of a 'worm'.

Table 2. *Variations in the length, circumference and radius of a cylinder of fixed volume*

(Volume (V) = 15,700 cubic units, r = radius, c = circumference, l = length.)

r	c	l	V
2	12·5	1260·0	15,700
3	18·9	555·6	15,700
4	25·1	312·4	15,700
5	**31·4**	**200·0**	**15,700**
6	37·7	138·9	15,700
7	43·9	102·1	15,700
8	50·3	78·0	15,700

The graph which can be drawn from the relation between 'l' and 'c' is shown in Fig. 3, and shows clearly that for a given percentage change in length of the circular muscles the longitudinals change in length by twice that percentage. Alternatively, for a given absolute change in length of the circular muscles the change in length of the longitudinals produced by it may be great or small according to whether l/r is at its greatest or smallest. An implication of this is that, since the smallest change in length of the animal for a fixed change in length of the circulars occurs when the animal is at its 'fattest', it would be expected that burrowing animals would carry out their burrowing when thick and not when thin. Conversely, when

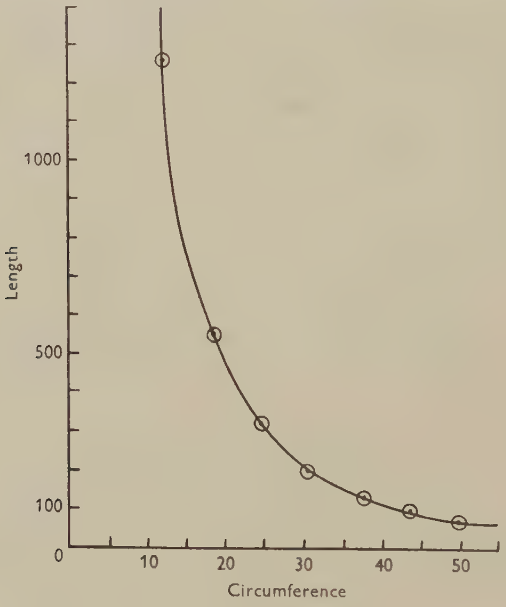

Fig. 3. Graph showing the relationship between the length and circumference of a cylinder of fixed volume.

an animal is crawling it will, for each fixed contraction of the circular muscles, progress farthest if it is thin. If, for example, the working range of an earthworm were 25% of its resting length, then, although this means a 50% change in length for the longitudinals, it involves only a normal working range of 25% for the circulars. Whether this difference in requirements is based on any difference in the physiology of the muscles is unknown.

VI. SUMMARY

Four aspects of the functioning of a fluid-filled cylindrical animal have been examined, viz.: (1) the role of the body fluid as a skeleton for the interaction of the longitudinal and circular muscles of which the animal must be composed; (2) the measurement of the maximum thrust which the animal can exert by measurement of its internal hydrostatic pressure; (3) the application of the force to the substratum and the part played by friction; (4) the relation between the changes in dimensions of the animal and the working length of the muscles.

Under (1) the necessity for a longitudinal and circular construction has been shown and the necessity for a closed system emphasized.

Under (2) the pressure exerted on the body fluid by the contraction of the longitudinal and circular muscles is discussed, and from their cross-sectional areas it is shown to be probable that when contracting maximally in *Lumbricus* they are not balanced, but that the longitudinals are about ten times as strong as the circulars.

Under (3) it is shown that the strength of an animal as measured by its internal hydrostatic pressure is sufficient to account for its customary activities. Use which may be made of the longitudinals during burrowing is pointed out.

Under (4) it is shown to be mechanically sound for burrowing animals of cylindrical form to be 'fat', but that a 'thin' animal is more efficient at progression.

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THE EFFECT OF PITUITRIN INJECTION ON THE WATER BALANCE OF *BUFO REGULARIS* REUSS.

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(*Received* 17 *August* 1949)

(With Three Text-figures)

I. INTRODUCTION

In 1921, Brunn showed that injection of neurohypophysial extracts causes a temporary increase in the body water content of Amphibia kept in water. Since then the existence of this water-balance effect has been confirmed by numerous workers, but its mechanism is by no means fully understood. The increase in body water following pituitrin injection might be due either to an increased rate of water uptake through the skin, or to a decreased rate of elimination of water through the kidneys, or to a combination of the two. Each type of effect has been claimed to have been demonstrated by various workers, but many of the experiments are open to criticism. It is convenient to consider separately the evidence for the existence of the two mechanisms.

(1) *Increased water uptake*

Brunn (1921) concluded that the water-balance effect was extra-renal in origin. He performed experiments on frogs in which the cloaca was ligatured. When kept in water pituitrin-injected animals were found to increase in weight more rapidly than uninjected controls, thus reflecting an increased rate of water uptake. After periods of 3, 6 and 8 hr. the ligature was untied, and the volume of urine that had been produced was measured. He found the same values for pituitrin injected as for normal animals, and therefore concluded that there was no renal effect. This latter conclusion is hardly justified, since normal urine production cannot be expected to occur with the cloaca blocked for long periods. Biasotti (1923), Steggerda (1931) and Rey (1935), in similar experiments, also demonstrated an increased rate of water uptake.

Novelli (1936), in experiments on toads, also claimed to have demonstrated an increased rate of water uptake in skin pouches with intact circulation. The pouches of pituitrin-injected animals were found to contain more fluid than those of uninjected controls. This finding, however, does not satisfactorily demonstrate an increased rate of water absorbtion; it might equally well result from a decrease in the rate of elimination of water from the pouches of the injected animals.

(2) *Decreased urine flow*

As mentioned above, Brunn's experiments do not warrant his conclusion that there is no decrease in urine production after pituitrin injection. Oehme (1919), and Burgess, Harvey & Marshall (1933) also failed to find any anti-diuretic effect.

On the other hand, Houssay & Potick (1929), Rey (1935), Adolph (1936) and Pasqualini (1938), in experiments similar to Brunn's, found a decreased rate of urine production following pituitrin injection. Howes (1940), using a dye-elimination method not involving cloacal ligature, also found an anti-diuretic effect. Howes points out that the water-balance effect in intact animals cannot be the result only of an increased rate of water uptake with no renal effect. The kidney is not normally functioning at its maximal rate, and therefore if renal physiology were unaffected the increase in water absorbtion would be compensated by an increase in urine production, and no gain in weight would occur.

There is thus considerable evidence in favour of the existence of both a renal and an extra-renal effect of neurohypophysial extracts. Unfortunately, however, most of the experiments involved the functional elimination of the kidneys, and are therefore open to the objection that the physiological condition of the animals was so abnormal as to render difficult the evaluation of the results obtained. Further experiments in which both the renal and the extra-renal effects can be studied simultaneously in a normal animal are therefore desirable. A method of cannulating the cloaca of toads has been devised, and this makes it possible to measure simultaneously both the uptake of water and the urine production. This technique has therefore been used to study the water-balance effect of mammalian posterior pituitary extract.

II. MATERIAL AND METHODS

The species used was *Bufo regularis* Reuss. The animals ranged in weight from 42 to 133 g. All experiments were carried out at 26° C. The pituitrin used was Parke Davis Pituitary (posterior lobe) Extract. The cannula consists of a piece of glass tubing with a slight bulb at one end, divided by a constriction from the main part of the tube (Fig. 1 *a*). The bulbed end of the cannula is inserted into the animal's cloaca and held in place by a cotton thread passing through the skin on the dorsal surface just above the anus, round the cloaca and out again through the skin close to where it entered (Fig. 1 *b*). The single stitch necessary to place the thread in this position is made with a fine curved surgical needle. The two ends of the thread are

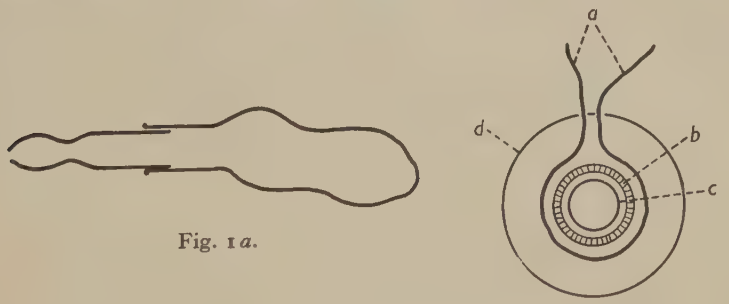


Fig. 1 *a*.

Fig. 1 *b*.

Fig. 1. (*a*) Cannula with balloon attached. (*b*) Diagrammatic cross-section of hindend of toad, showing mode of attachment of cannula. *a*, loose ends of thread ready for tying; *b*, cloacal wall; *c*, cannula; *d*, skin of toad.

then tied tightly in such a way that the thread firmly holds the constriction in the cannula in position in the cloaca. A toy balloon is attached to the end of the cannula, and in this the urine is collected. In preliminary trials it was found that the animals showed no ill effects as a result of this treatment.

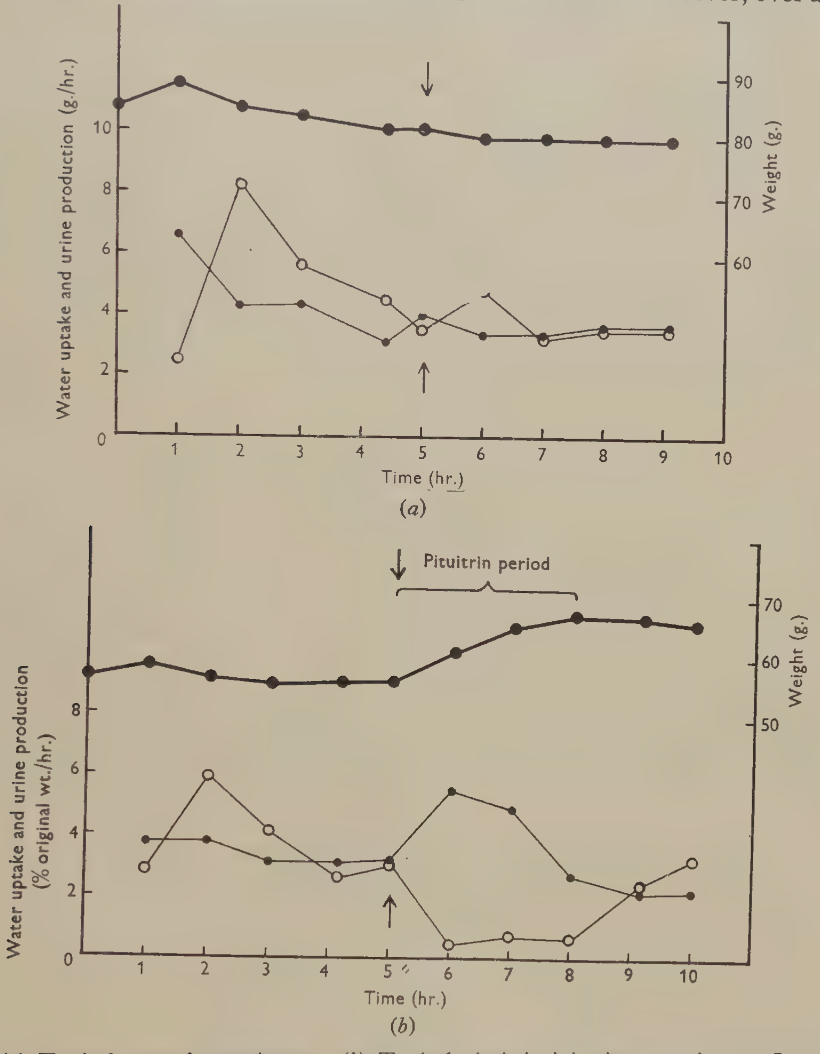
The toads were kept without food, but with access to water, for a few days before an experiment. This allowed the gut to be emptied and thus prevented complication due to the elimination of faeces during an experiment. The procedure was as follows. The toad was placed in a covered dish of water, kept in a thermostatically controlled water-bath, for 1 hr. The depth of water was such as to cover the greater part of the toad's body, but allowed it to rest with its head above water. This period allowed the animal to adjust to the experimental temperature and reach its equilibrium water content. The animal was then removed from the water, the cannula inserted under light ether narcosis, and the animal allowed to recover. It was then dried carefully, weighed and returned to the water-bath. At approximately hourly intervals the animal was removed, dried and weighed, the increase in weight giving the water uptake during the previous period. The balloon was then removed from the cannula, emptied, replaced and the animal weighed again. The amount of urine voided each hour, as well as the total water uptake, was thus known. After five such hourly weighings pituitrin was injected into the crural lymph sac and the experiment continued. As will be seen from the results quoted later, the injection was followed by a weight increase which reached its maximum in 3 or 4 hr., and thereafter recovery started. In the first experiments weighings were continued until recovery was complete, and the original weight had been regained, but later this was considered unnecessary, and the experiments were stopped as soon as the weight began to decrease.

In the control series the procedure was as before, except that the injection made after the first 5 hr. consisted of mammalian Ringer-Locke solution. Controls and pituitrin-injection experiments were run alternately, with the experimental and control animals as far as possible paired for size, sex and general condition. In each case the volume of Ringer injected into the controls was the same as that of the pituitrin used for the corresponding experimental animal.

III. RESULTS

(1) *General*

Fig. 2*a* shows a typical control experiment. The weight of the animal remains more or less constant with only minor oscillations. The rate of water uptake is very high in the first hour; this was frequently, but not invariably, the case, and is probably due to incomplete recovery from the anaesthetic. It was observed that the ether caused sufficient peripheral vasodilation for there to be visible flushing of the skin. If recovery from this effect were not complete when the experiment was started a high initial water uptake might well result. For this reason it was decided to discard the first hour's readings in all calculations. Apart from this initial high value there is a tendency, more marked in some experiments than in others, for the rate of water uptake to show a gradual decline during an experiment. The large oscillations in urine production are probably not due to real variations of such magnitude in the rate of urine formation, since the toad does not always empty its bladder just before being weighed. The urine collected in the balloon at each weighing is therefore not necessarily equal to the urine produced in the previous hour. However, over a period of several hours the irregularities due to this factor will tend to even themselves out, and a reasonable measure of the rate of urine production may be obtained.



(*a*)

(*b*)

Fig. 2. (*a*) Typical control experiment. (*b*) Typical pituitrin injection experiment. Large filled circles, weight of toad (right-hand scale); small filled circles, rate of water uptake in previous period; small open circles, rate of urine production in previous period. The arrows indicate the times at which the injections were made.

Fig. 2*b* shows the results of a typical pituitrin-injection experiment. The weight remains more or less constant throughout the first period, but after the injection of pituitrin it increases markedly, reaching a maximum after 3 hr., and then begins to decrease. The urine production shows a sharp fall after injection, and later a rise which ultimately brings the animal's weight back to normal. In the pre-injection period the water uptake shows the usual slight fall, but injection is followed by an increase, after which the value gradually returns to slightly less than its original level.

It was decided to treat the results as follows. The 'pituitrin period' is taken as the period from the time of injection until the weight is at a maximum. Recovery is taken as having started as soon as the animal's weight starts to decrease. The rate of water uptake per hour is calculated for the whole of the pituitrin period, and then expressed as a percentage of the rate of uptake during the pre-injection period, exclusive of the first hour. The urine production is treated in the same way. These values will be referred to as the relative water uptake and relative urine production. In the control experiments the rates of water uptake and urine production in the post-injection period are similarly expressed as percentages of the corresponding rates in the pre-injection period, excluding the first hour. In any control experiment the post-injection period is taken as being the same length of time as the pituitrin period for the corresponding experimental animal.

(2) *The effect of pituitrin on water uptake*

In the first experiments the dosage of pituitrin was not controlled, 5 i.u. (0·5 ml.) were injected, irrespective of the weight of the toad. It was soon found that the results were very variable. Although after pituitrin injection there was always a weight increase and a diminished urine flow, the rate of water uptake sometimes increased and sometimes showed the slight decrease which was usual in the controls The variability seemed to be a function of dosage, and therefore in subsequent experiments the amount of pituitrin injected was varied so that the dosage covered a range of 1·9–15·3 i.u. per 100 g. body weight. Table 1 shows the relative rates of water uptake for all the pituitrin dosages used, together with the values found in the control series. Fig. 3 shows the same results graphically. Although the variability

Table 1. *Relative rates of water uptake of pituitrin-injected toads, and of saline-injected controls*

Low dosage		High dosage		Controls
Dosage (i.u./100 g.)	Relative water uptake	Dosage (i.u./100 g.)	Relative water uptake	Relative water uptake
1·9	88	8·5	145	76
2·1	88	8·8	134	74
3·4	171	8·9	117	83
3·7	94	10·0	123	80
4·5	98	11·8	210	86
5·5	116	12·0	145	96
6·3	56	13·5	161	101
6·8	100	15·3	115	106
7·5	76			92
8·2	68			
Mean	95·50 ± 9·93		143·75 ± 10·99	88·2 ± 3·74

is considerable, it is clear that water uptake is consistently increased by pituitrin only when the dosage is high, while low dosages are often without effect. The experiments were therefore divided into two groups, one with dosages of up to 8·4 i.u./100 g., the other 8·5–15·3 i.u./100 g. In each category the mean relative rate of water uptake was calculated and compared with the value found for the controls. With a dosage less than 8·5 i.u./100 g. the rate is 95·5 ± 9·93, while for the controls the value is 88·2 ± 3·74. These two values are not significantly different. The pituitrin-injected animals include one which gave an extraordinarily large effect, 171 %. If this value is neglected then the mean relative water uptake for dosages of less than 8·5 i.u./100 g. becomes 87·11 ± 6·04, which is very close to the value found for the controls. We can therefore conclude that, apart from the one exceptional animal which had a very high sensitivity to pituitrin,* dosages of pituitrin less than 8·5 i.u./100 g. have no effect on the rate of water uptake.

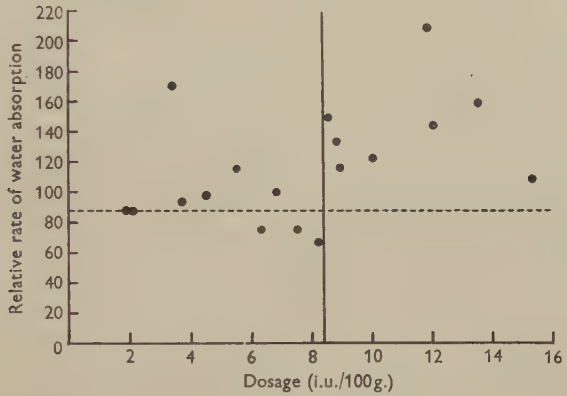


Fig. 3. Relation of rate of water uptake to pituitrin dosage. The relative water uptake is the rate of water uptake during the pituitrin period expressed as a percentage of the rate in the pre-injection period. The broken line represents the value for the controls, and the vertical line represents the point at which the division into low and high dosages was made.

For dosages of 85 i.u./100 g. and over, the mean relative rate of water uptake is 143·75 ± 10·99. This differs significantly from the control value. A dosage of 8·5 i.u./100 g. or over therefore causes a significant increase in the rate of water uptake.

(3) *The effect of pituitrin on urine formation*

Table 2 shows the relative rates of urine production at low and at high dosages of pituitrin, together with the values found for the controls. The mean values for the urine production at the two dosages are 26·20 ± 3·17 and 26·25 ± 4·78 respectively, while the control value is 79·2 ± 5·46. The rates at the two dosages do not differ significantly from each other, but both are significantly less than the control value. Pituitrin injection at all the dosages used thus has an anti-diuretic effect.

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* Moulting in Amphibia is controlled by the posterior pituitary. This animal may have been about to moult and may therefore have had an unusually high concentration of pituitary hormones in its blood.

Table 2. *Relative rates of urine production of pituitrin-injected toads, and of saline-injected controls*

Low dosage		High dosage		Controls
Dosage (i.u./100 g.)	Relative urine production	Dosage (i.u./100 g.)	Relative urine production	Relative urine production
1·9	13	8·5	26	68
2·1	25	8·8	17	71
3·4	48	8·9	21	56
3·7	25	10·0	8	70
4·5	37	11·8	44	111
5·5	19	12·0	44	78
6·3	25	13·5	15	92
6·8	23	15·3	35	91
7·5	29			76
8·2	18			
Mean	26·20 ± 3·17		26·25 ± 4·78	79·2 ± 5·46

(4) *The distribution of the excess water*

During the course of the experiments it was noticed that in animals which had taken up excess water after injection the lymph sacs were extremely full of fluid. It therefore seemed desirable to find out whether most of the excess water was retained in the lymph spaces, or whether it was more or less uniformly distributed throughout the tissues. For this purpose toads were placed in the water dish for an hour to equilibrate. They were then removed, injected with 5 i.u. of pituitrin, weighed and replaced in the water-bath for a further period of 2 hr. They were then dried and weighed again to find the percentage weight increase. In the eleven determinations made the mean weight increase after injection was 16·55 %. The animals were then killed rapidly with ether, and certain tissues removed for water content determinations. The tissues used were skin, muscle, gut and liver. The whole skin was not used, but only the part from the 'waist' down. The muscles were the two gastrocnemii. The gut was removed by two cuts, one through the oesophagus just above the stomach, the other through the distal end of the rectum. The stomach was slit open and blotted dry on both surfaces, while the rest of the gut was emptied of its contents by squeezing under the blunt edge of a scalpel blade. The tissues were weighed as quickly as possible, after blotting off the surface fluid, and then dried at just over 100° C. for 3 days and reweighed after cooling. In a control series the normal water content of the tissues was determined in the same way after the animal had remained in the water-bath for the usual hour's equilibration period.

The results are given in Table 3. In the case of skin and liver the differences between controls and injected animals are not significant, in the case of gut the significance is doubtful, while for the muscles the difference is significant. The magnitude of the effect, however, is very small, less than 3 %, while the mean increase in body weight of the animals from which the tissues had been taken was 16·55 %. Thus, although the increase in body weight is considerable, the degree of tissue hydration is very slight. An attempt was made to assess the degree of dilution of the blood, by making erythrocyte counts before injection and just before killing. The first sample can be satisfactorily withdrawn from the mandibular artery without subsequent bleeding. It was found, however, that it was impossible to obtain a satisfactory second sample. The waterlogged state of the lymph spaces made it impossible to withdraw a sample uncontaminated with lymph, and the blood counts obtained were therefore artificially low. In spite of this extra dilution the values obtained showed a mean percentage dilution which was approximately equal to the percentage increase in water content of the whole animals. The real degree of dilution of the blood, as a result of the increased water uptake following injection, must therefore have been considerably less. Since the excess water is thus not accounted for either in the blood or other tissues it is clear that most of it is retained in the lymph spaces. This implies a type of internal osmotic regulation, but the mechanism by which it is achieved has not yet been studied.

Table 3. *Percentage water contents of tissues of pituitrin-injected and of normal toads*

(Each value represents the mean of ten determinations in the control series, and eleven in the pituitrin-injected animals.)

	Injected	Controls
Skin	76·6 ± 0·48	76·1 ± 0·50
Muscles	80·8 ± 0·56	78·5 ± 0·53
Gut	81·7 ± 0·50	79·9 ± 0·79
Liver	64·7 ± 2·80	67·3 ± 2·12

IV. DISCUSSION

The experiments described above have shown that in *Bufo regularis* the water-balance effect is the result both of an increased uptake and a diminished output of water. Using mammalian posterior pituitary extract the latter effect is seen to be produced at lower dosages than the former. This could be interpreted as a difference in thresholds for response to a single substance, or equally well as indicating that the two types of response are elicited by distinct substances, present in widely different concentrations in the extract used. Heller (1945) assumes that a single substance is responsible for the two types of effect, while Houssay (1949) assumes that two are involved. No critical evidence exists to decide the question. Previous work on the active principle responsible for producing the water-balance effect is difficult to evaluate, since the effect has generally been treated as a unit, without differentiation into renal and dermal components. There is agreement that pitocin produces a greater effect than pitressin (Heller, 1930*b*; Steggerda & Essex, 1934; Oldham, 1936; Boyd & Brown, 1938). This has usually been interpreted as indicating that the oxytocic fraction either contains or is itself the factor causing the amphibian water-balance effect. This is an unjustifiable conclusion. The workers mentioned above, although they found pitocin the more effective, did not find pitressin devoid of activity: moreover Heller & Smith (1948) obtained from crab's eye-stalks an extract devoid of oxytocic power which produces a water-balance effect in *Rana* *temporaria*. It is not possible to reach any conclusion until the effects of the oxytocic and the pressor fractions have been tested, using a method which differentiates between the renal and the dermal components of the water-balance effect.

The mechanisms of the two components of the water-balance effect are not understood. Houssay (1949) implies that the anti-diuretic effect is the result of increased tubular resorbtion, just as it is in mammals, but little evidence is adduced to support this view. Posterior pituitary extracts have been shown to cause anti-diuresis in reptiles by constriction of the glomerular arterioles (Burgess *et al.* 1933), and also to cause constriction of the glomerular vessels in the frog's kidney (Richards & Smith, 1924; Adolph, 1936; Pasqualini, 1938). It therefore seems more probable that the anti-diuretic effect in Amphibia results from a reduction in glomerular filtration, rather than from an increase in tubular resorbtion.

The increased rate of water uptake after pituitrin injection may result either directly from changes in the skin permeability, or indirectly as a consequence of dilation of the skin capillaries. In favour of the latter view are Howes's (1940) experiments, showing that the water-balance effect is absent in tadpoles of *Bufo bufo bufo*, and appears gradually, *pari passu* with the development of the skin capillaries. It should, however, be noted that Howes did not differentiate between the renal and dermal components of the water-balance effect, and makes no mention of the relation of the magnitude of the effect to the stage of development of the kidney.

The results obtained on the distribution of the excess water indicate that most of it is retained in the lymph spaces, and that the tissues are protected against excessive hydration. Heller (1930*a*), working with *Rana esculenta*, obtained similar results, the degree of hydration of the tissues being somewhat higher than I have found in *Bufo regularis*. Although the excess water load is, of course, abnormal, the regulative mechanism controlling its distribution may be of considerable significance in normal conditions. The body water in Anura can vary very widely. When the animal is in contact with water the lymph spaces are filled, and when it leaves water it thus has a considerable reserve which can be allowed to evaporate with little effect on the tissues. For this water to be kept available for evaporation it is essential that it should not simply be eliminated by the kidneys. This implies some sort of internal osmotic regulation which, presumably, involves the integrated control of the blood pressure, the activity of the lymph hearts and the capillary permeability. Further work is necessary before this can be regarded as more than a hypothesis, but if it is correct then the adaptive significance of the peculiar organization of the anuran lymphatic system becomes clear. Its function is to maintain a 'reserve tank', in which water can be stored temporarily and allowed to evaporate gradually when the animal is on land, without greatly influencing the water content of the tissues of the body.

V. SUMMARY

1. A method of cannulating the cloaca of Anura is described.
2. Using this method it is found that in *Bufo regularis* the increase in body water which follows pituitrin injection is the result of an increased rate of water uptake, together with a diminished urine flow.

3. The excess water is mostly retained in the lymph spaces, while the water content of the tissues of the body increases very little.

4. The bearing of these results on the problem of the identification of the substance or substances which elicit the amphibian water balance effect, and the biological significance of the organization of the anuran lymphatic system are discussed.

A brief account of this work has already been published (Ewer, 1949). In this account there is an error, the figures given for the water contents of gut and liver have been transposed.

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THE EFFECT OF POSTERIOR LOBE PITUITARY EXTRACTS ON THE BLOOD PRESSURE OF *ORNITHORHYNCHUS* (DUCK-BILLED PLATYPUS)

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(With Four Text-figures)

I. INTRODUCTION

Whole extract of posterior lobe pituitary has several pharmacological properties (Waring & Landgrebe, 1950). Three of these are relevant to the present theme:

(*a*) Intravenous injection into spinal or fully anaesthetized eutherian mammals evokes a *rise* of blood pressure.

(*b*) Intravenous injection into spinal or anaesthetized fowls or ducks evokes a *fall* of blood pressure.

(*c*) After injection by any route (or *in vitro*) the uterus contracts.

Since no chemically pure excitant has been extracted for certain* from the posterior lobe, activity of a sample is expressed in International Units which are arrived at by direct comparison of the unknown extract with International Standard powder on a generally agreed biological preparation. Here it need only be noted that 'pressor' units are assayed on spinal or anaesthetized mammals, and 'oxytocic' units are assayed on the guinea-pig uterus *in vitro*.

By definition, 0·5 mg. International Standard powder contains 1 unit of oxytocic and 1 unit of pressor activity. In what follows, reference to pressor and oxytocic units are strictly to these activities as measured under the conditions specified in Mem. 36 and does not refer generally to blood-pressure raising, or uterus-contracting properties.

By fractional precipitation, or fractional absorption and elution, two fractions can be obtained from a simple whole extract. One, variously called oxytocin, pitocin, post lobin O, has oxytocic properties contaminated with 3–5% pressor activity. The other, variously called vasopressin, pitressin, post-lobin V, has pressor properties contaminated with 3–5% oxytocic properties.

Of the effects listed above (*a*) is evoked by pitressin and not to any measurable extent, by pitocin, (*b*) is evoked by pitocin predominantly (see p. 56) and (*c*) is evoked by pitocin; pitressin has no effect on the uterus *in vitro* unless there is magnesium in the saline. Since histamine, a common contaminant of pituitary extracts causes contraction of the uterus and depression of the blood pressure, it is relevant to note that the above observations have been made using histamine-free extracts.

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* It is possible that Van Dyke's preparation (1942) is pure, but this is not generally available.

The mammalian pressor response results from peripheral vasoconstriction, and experiments involving gross exclusion of various organs show that the effect is not localized to any particular organ or organs. Moreover, since some peripheral vessels constrict while others dilate, this implies that the pressor effect is not a simple one, and, of course, the orthodox measurement with a cannula in the carotid gives only an over-all figure. Generally speaking, pituitrin has no general effect on the heart, so the rise in pressure must be attributed to the heart's increased response to the load imposed by peripheral vasoconstriction. Paton & Watson (1912), who were the first to describe the avian depressor response, showed by oncometer recordings that it was accompanied by vascular dilatation in the splanchnic area, but Hogben & Schlapp (1924) showed that the response was still exhibited when this area was excluded from circulation.

Although much work remains to be done on the exact mechanism of the responses evinced by birds and mammals, the existence of such a gross difference in their over-all response to whole posterior lobe, and its separated fractions, is challenging with regard to the phyletic distribution of the two responses. Work shortly to be published will show that in some living reptiles, there is a pressor response to pitressin, a depressor response to pitocin and a depression to whole extract; the same is substantially true for the bird. So, in attempting a phyletic formulation comparable observations on a prototherian are of pivotal importance.

II. EXPERIMENTAL

Blood pressure was recorded in the usual way with a mercury manometer and a cannula inserted in the carotid. Injections were made into the femoral vein, each injection being washed in with 1 c.c. of saline. Control injections of saline were also 1 c.c. Heparin was used as anticoagulant in the animal and citrate in the manometer.

Two males and one female were obtained, each weighing between 2 and 3 kg. Each was prepared so as to exhibit a different level of central nervous control with the object of obtaining the maximum information for comparison with known data from birds and eutherians. As has been indicated, the degree of central nervous control in eutherian preparations is correlated only with quantitative changes in the response evoked. In the bird, however, we have reason to suspect that depth of anaesthesia, pithing, etc., may to some extent determine the qualitative nature of the response (see discussion later). The three preparations were therefore designed to produce the following states of central depression: (*a*) anaesthetized, respiratory centre functional (preparation 1); (*b*) anaesthetized, respiratory centre inhibited (preparation 3); (*c*) spinal (preparation 2).

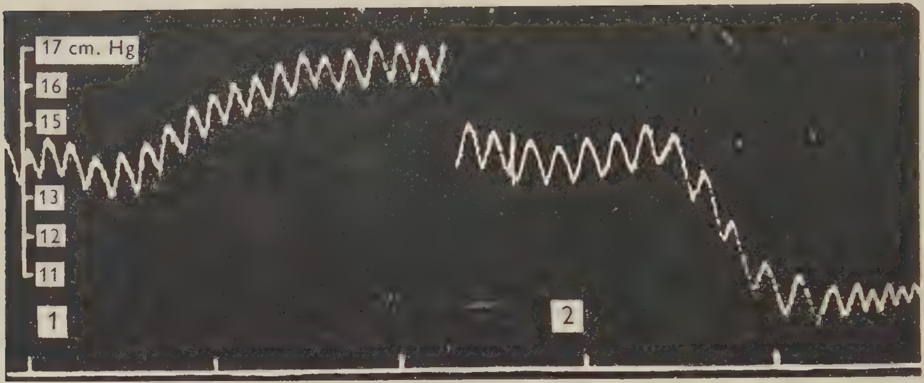

Fig. 1. Preparation 1. 1, 0·5 i.u. of pitressin; 2, 0·5 i.u. of pitocin. Time marks at minute intervals.

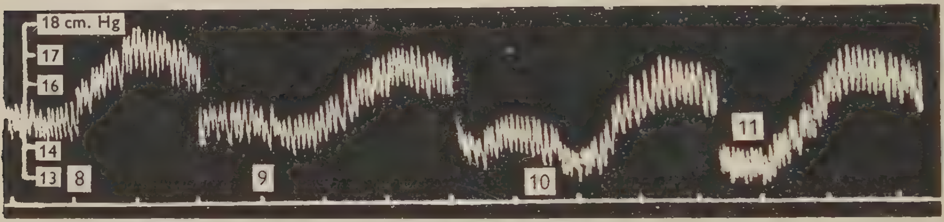

Fig. 2. Preparation 1. 8, 0·6 i.u. pitressin; 9, 0·6 i.u. pitressin; 10, 0·6 i.u. pitressin; 11, 1·2 i.u. pitressin. Time marks at minute intervals.

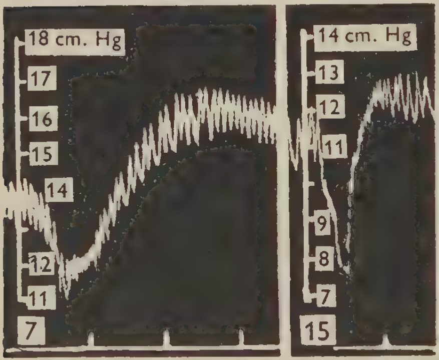

Fig. 3. Prepartion 1. 7, 0·6 i.u. pitressin + 0·6 i.u. pitocin; 15, unfractionated posterior lobe pituitary extract. Time marks at minute intervals.

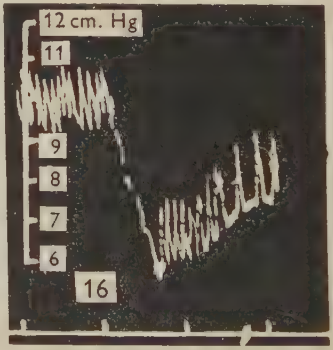

Fig. 4. Preparation 1. 16, 0·05 mg. (approx.) histamine acid phosphate. Time marks at minute intervals.

Preparation 1. *Male—21 January* 1949*—working sheet*

Animal anaesthetized with intraperitoneal Dial and urethane; vagi severed; trachea cannulated; no artificial respiration.

Time	Ref. no.	Injection	Response	Increment or Decrement (cm.)	Notes
17·45	—	0·25 c.c. 'Dial' (Ciba liquid)	—	—	Intraperitoneal injection
18·40	—	0·1 c.c. 'Dial' ,, ,,	—	—	—
20·30	—	200 mg. urethane	—	—	—
20·45	—	—	—	—	Dissection commenced
21·00	—	200 mg. urethane	—	—	—
21·15	—	200 mg. urethane	—	—	—
21·40	—	0·1 c.c. 'Dial'	—	—	—
22·05	—	—	—	—	Cannulae inserted
22·10	—	heparin	—	—	Intravenous injections
22·12	—	1 c.c. 0·9 % saline*	13·0–13·5	Up 0·5	Drum speed 48 mm./min.
23·50	1	0·5 i.u. pitressin	13·0–16·0	Up 3·0	—
00·07	2	0·5 i.u. pitocin	13·0– 9·4	Down 3·6	—
00·24	3	0·6 i.u. pitocin	13·0–10·6	Down 2·4	Drum speed 10 mm./min.
00·34	4	0·6 i.u. pitressin	13·0–15·8	Up 2·8	—
00·48	5	0·5 i.u. pitocin	13·0–10·8	Down 2·2	—
		+0·5 i.u. pitressin	13·0–15·4	and up 2·4	—
01·07	7	0·6 i.u. pitocin	12·6–10·4	Down 2·2	—
		+0·6 i.u. pitressin	12·6–15·6	and up 3·0	—
01·15	8	0·6 i.u. pitressin	14·0–16·2	Up 2·2	—
01·24	9	0·6 i.u. pitressin	14·0–16·0	Up 2·0	—
01·31	10	0·6 i.u. pitressin	12·8–12·6	Down 0·2	—
			12·8–15·0	and up 2·2	—
01·40	11	1·2 i.u. pitressin	12·8–15·4	Up 2·6	—
01·48	12	1·2 i.u. pitressin	12·8–15·0	Up 2·2	Leakage from venous cannula and movement of animal
01·56	13	1·2 i.u. pitressin	12·8–14·4	Up 1·6	—
02·02	14	Unfractionated P.L.P.	12·0–10·0	Down 2·0	—
			12·0–15·6	and up 3·6	—
02·10	15	Unfractionated P.L.P.	10·4– 7·6	Down 2·8	—
			10·4–12·0	and up 1·6	—
02·18	16	0·05 mg. (approx.) histamine acid phosphate	9·5– 5·9	Down 3·6	—
02·20		—	—	—	Died

* Note: 1 c.c. saline injected before every pituitary injection. Except in the case of the first injection these evoked no response.

Conclusions and comments from experiment 1

(1) Response to histamine-free pressor fraction (pitressin) is similar to that of an eutherian mammal (i.e. a simple slow rise). The amount of excitant needed to evoke a given rise is roughly ten times that needed to evoke an equivalent rise in a cat or rabbit similarly anaesthetized.

(2) Comparison of rises to similar doses from a base line of 13 and 14 cm. shows the greater sensitivity of the animal at the former level. Serial doses (9–12) show that *at the arbitary level* chosen there is poor discrimination and no evidence of a strong tolerance developing. Since, of course, a dose-response curve is never straight, it might well be that at another level there would be good discrimination.

(3) Response to histamine-free oxytocic fraction (pitocin) is similar to that of the bird (i.e. a swift depression) except that the depression is more prolonged. The amount of excitant needed is roughly ten times that needed to evoke an equivalent depression in the anaesthetized bird. We have insufficient data to comment on either discrimination between doses of pitocin or the development of tolerance.

(4) There is a depressor response to histamine. Unfortunately our weighed tubes of solid histamine acid phosphate were broken in transit and in the semi-field conditions under which we were working, the dose could only be estimated visually. So the result is only of qualitative value.

(5) The response to mixtures of pitressin and pitocin was a depression followed by a rise. The only two injections (nos. 5 and 7) made with this mixture seemed to show that neither constituent seriously interferes with the effect of the other, both fall and rise being manifested.

(6) The whole question of the unitary or multiple nature of the excitant from the posterior lobe has been reviewed recently by Waring & Landgrebe (1950). In view of the evidence marshalled by them for the close bonding of pressor and oxytocic activities in unfractionated extracts, it is important to know whether the response to unfractionated extract is exactly similar to that elicited by a mixture of the chemically separated activities. For reasons set out in no. 4 above we could not inject carefully measured doses. The injections used (nos. 14 and 15) were of freshly prepared extract from dried glandular material (containing no more than the equivalent of 0·2% histamine acid phosphate) and were, in the absence of a delicate balance, judged visually for quantity. It is evident that the type of response was indistinguishable from that evoked by a mixture of the two separated fractions.

(7) Injection of saline alone had insignificant effects.

(8) In our experience with eutherian mammals, the rise to injections of histamine-free pressor fractions is never preceded by a depression, under properly controlled conditions. If there is a depression it is only transitory and attributable to (*a*) too light anaesthesia, or (*b*) in occasional cases, to preparations very sensitive to the rapid injection of cold saline. In the experiments described here, there was no preliminary fall preceding the rise to pitressin; there was no response to saline save a small and very transitory rise; and there was no histamine injected. Therefore the fall to pitocin and to pitocin-pitressin mixtures must have been due to a pituitary principle.

Conclusions and comments from experiment 2

This preparation exhibited the fall to pitocin and rise to pitressin in a manner similar to the first preparation. It was unmistakably less sensitive to pitressin than the first, and this can probably be attributed to the low base-line (3–4·5 cm.). We have previously found in other animals that below a certain base pressure, the response exhibited is very much less than with a slightly higher pressure; in the rat (Landgrebe, Macauley & Waring, 1946) this threshold is 4 cm. Hg. In the same way, of course, the response is reduced by a high base pressure attained by light anaesthesia or transection of the brain anterior to the medulla. For the same reason the small depression to pitocin is not surprising and is consistent with our experience with the bird.

Preparation 2. *Male—*22–23 *January* 1949*—working sheet*

Animal etherized; vagi severed; trachea cannulated; spinal cord severed at level of the axis vertebra, and brain destroyed in othodox fashion (see Burn, 1937). Artificial respiration; pump speed 40 strokes per min. No atropine administered.

Time	Ref. no.	injections, etc.	Response	increment or or decrement (cm.)	Notes
18·00	—	Ether	—	—	—
19·55	—	Pithed, artificial respiration	—	—	Pump speed 40/min. cannulae inserted intravenous injections
20·00	—	—	—	—	—
20·15	—	heparin	—	—	—
21·00	1	0·5 i.u. pitressin	3·0–4·2	Up 1·2	—
21·03	2	0·6 i.u. pitocin	4·6–3·6	Down 1·0	—
21·13	3	0·1 c.c. adrenalin (1 : 100,000)	4·6–5·4	Up 0·8	—
21·16	4	0·2 c.c. adrenalin (1 : 100,000)	4·6–6·6	Up 2·0	—
21·20	5	1·0 i.u. pitressin	4·6–7·0	Up 2·4	—
21·28	6	1·0 i.u. pitressin	6·2–8·4	Up 2·2	—
21·42	7	1·0 i.u. pitressin	6·2–7·3	Up 1·1	—
21·47	8	1·0 i.u. pitressin	6·2–7·0	Up 0·8	—
32·52	9	1·0 i.u. pitressin	6·2–7·4	Up 1·2	—
22·10	10	1·0 i.u. pitocin	—	—	Carotid cannula slipped so no response recorded
22·14	11	1·0 i.u. pitocin	4·0–3·6	Down 0·4	—
22·16	12	1·0 i.u. pitocin	—	—	No response
22·18	13	1·0 i.u. pitressin	4·0–4·8	Up 0·8	—
22·44	14	1·0 i.u. pitocin	3·0–2·4	Down 0·6	—

Preparation 3. *Female—*23 *January* 1949*— working sheet*

This animal was initially anaesthetized with intraperitoneal Dial supplemented by urethane. The procedure described for preparation 1 was followed and the rise to pitressin and fall to pitocin confirmed. The blood pressure of this animal was consistently 4 cm. Hg and, as was to be expected, neither the rise nor the fall to appropriate stimulation was as great as in the first preparation. We then tried to inhibit the respiratory centre by intravenous injection of soluble pentobarbitone. This was accomplished, the blood pressure fell to 3 cm. and the animal was successfully maintained with artificial respiration, but we were unable subsequently to elicit trustworthy responses with any pituitary preparation.

III. DISCUSSION

With only three animals available we tried to set up the three classes of preparation for which there are comparable data from Eutheria, viz. (*a*) fully anaesthetized with respiratory centre functioning; (*b*) spinal, with artificial respiration; and (*c*) fully anaesthetized with respiratory centre inactivated and artificial respiration. We were very fortunate with respect to (*a*), and sufficient information was obtained from (*b*) to show that in essential respects its behaviour was similar to (*a*). (*c*) was a failure.

We may note in passing the very high blood pressure exhibited by (*a*), but if this has significance it escapes us.

Eutherian mammals treated as either (*a*) or (*b*) exhibit a simple rise in blood pressure to either whole posterior pituitary extract or the pressor fraction. Moderate doses of pitocin* exert no effect measurable by the usual mercury manometer. Injection of enormous doses of pitocin result in a pressor response due to unmasking of the slight pressor contamination.

The chief interest of the present findings is therefore the depressor action of the oxytocic fraction, and the combined depressor-pressor action of whole extract. We have no clue to the seat of action of either, and it therefore remains an open question as to whether both excitants activate the same effectors in opposite ways, or whether their effects are separately localized. In either case the tracings make it clear that the depressor mechanism is more quickly activated that the pressor.

In exploring the phyletic distribution of these two mechanisms we look naturally to the birds and reptiles. A fair amount of information has been published about avian responses, but most work on this subject has been directed towards development of a reliable oxytocic assay, and consequently does not fully answer all the questions we wish to pose. Information about reptilian responses is meagre.

Paton & Watson (1912) first described the depressor response of the decapitate duck to whole pituitary extract. Their tracings show a *simple* precipitate depression. Hogben & Schlapp (1924) confirmed their findings and showed that the effect was evoked by histamine-free extracts and was therefore a pituitary action. Their tracings commonly show a simple fall. When separated fractions became available, Gaddum (1928) showed that the depressor response was attributable to the oxytocic fraction, but he gave no information about the effects of pitressin or of mixtures of pitressin and pitocin.

Coon (1939), Smith (1942), and Smith & Vos (1943) have developed the depressor response of the bird as a biological assay to pitocin, but due to the orientation of their studies they present (to us) a rather confusing picture of the relation of effects evoked by pitocin and pitressin. This much seems clear from their work:

(1) Injections of pitocin evoke a simple fall followed by a very slight rise.

(2) Tolerance is rapidly developed to large doses of pitocin (no fall evoked).

(3) In birds not tolerant to pituitary, pitressin evokes a simple fall and slight secondary rise (greater than pitocin secondary rise).

(4) Pitressin into oxytocic-tolerant fowl evokes 'pure rise' (Coon's words).

(5) Pitocin + pitressin ($P{:}O<3{\cdot}5{:}1$) evokes a simple fall equivalent to that which would have been evoked by oxytocic content alone.

(6) Pitocin + pitressin ($P{:}O>3{\cdot}5{:}1$) evokes a fall greater than that attributable to oxytocic content.

These results indicate that the bird response is unique, but work in progress shows that this is not so for all bird preparations. In a fowl heavily anaesthetized with phenobarbitone (respiratory centre inhibited) we have obtained a pure rise to pitressin and a fall and subsequent rise to pitocin. Since this rise can be obtained with the first injection, the effect is not to be attributed to pitocin tolerance. We have also obtained a consistent rise to pitressin and fall to pitocin in pigeons heavily anaesthetized with pentobarbitone.

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* With high doses of pitressin, the coronary constrictor action of the drug is shown by transitory heart failure; this may be masked or alleviated if pitocin, which has a dilator effect on coronary arteries, is also present.

All this needs further analysis, but a simple interpretation and comparison that fits the facts at present is: (*a*) that in the platypus (under the conditions described) and in deeply anaesthetized birds in our hands there is a simple depression to pitocin and a simple rise to pitressin; (*b*) that with a mixture of pitressin and pitocin (and possibly unfractionated extract) the two effects of these excitants follow serially, and while this reaction is simple in the platypus it is complicated in the bird by the double effect of pitressin which first potentiates the depression and later exhibits its own pressor effect in reduced degree.

The scanty information about the effect of posterior lobe extracts on reptiles is as follows: Hogben *et al.* (1924) found that with unfractionated extract Chelonians show a simple slow fall of little magnitude; the present authors (unpublished) have found that in the lizard, *Trachysaurus rugosus*, depression can be elicited by pitocin and a rise by pitressin.

Finally, we may be permitted to speculate about the phylogenetic significance of our findings, irrespective of whether our interpretation of the sequence of events in birds and platypus will stand up to later observation. In the evolutionary dichotomy from reptiles which produced the birds on the one hand, and the eutherian mammals on the other, the avian stem retained both mechanisms (though somewhat complicated), whereas the eutherian stem has lost its depressor mechanism. If we assume that the usual reptilian condition is to have both the pitocin-depressor and the pitressin-pressor mechanism, then in this respect the monotremes show a closer affinity to the sauropsidan stock than to the eutherian. Unfortunately, all therapsids are extinct.

When these observations were completed we heard indirectly that Prof. Matthey of the University of Lausanne had been making investigations on the chromosomes of the platypus, and in response to a request for his general conclusions he courteously wrote us: 'je n'ai que des observations très incomplètes sur les chromosomes de Platypus, mais qui confirment complètement celles de White, sur l'Echidne. Les chromosomes sont de types "Oiseau" ou Tortue. Très nombreux (60–80), distribuès en macro—et en microchromosomes. Si fragmentaires que soient ces observations, elles suffisent pour affirmer que la parente des Monotrèmes avec les Marsupiaux (cf. Gregory, 1940) est exclue totalement. Ils doivent representer un rèmeau independant au voisinage des Oiseaux et des Cheloniens.' Thus our inferences from pharmacological data receive support from an entirely different source.

IV. SUMMARY

1. The carotid blood-pressure response to intravenous injection of histamine-free extracts of mammalian posterior pituitary extracts in the platypus (*Ornithorhynchus*) is described.

2. Pitressin evokes a simple pressor response.

3. Pitocin evokes a simple depressor response.

4. Mixtures of pitressin and pitocin evoke a fall and a subsequent rise.

5. Histamine evokes a depressor response.

6. A preliminary note is made of the simple pressor response to pitressin and depression to pitocin in the deeply-anaesthetized bird and in a lizard.

7. Comparison is made between the responses to posterior pituitary extracts in reptiles, birds, the platypus and eutheria.

8. The possible phylogenetic significance of these observations is discussed.

This work required considerable organization because of the difficulty of obtaining living material. We are heavily indebted to the following individuals: Mr K. W. Taplin for help in converting electrical equipment; Prof. D. Wright for the loan of kymographic apparatus; Mr F. L. Combes and his assistants of the Tasmanian Wild Life Preservation Department; and finally, Prof. V. V. Hickman for generous hospitality at his home and laboratories in Hobart and for his constant support in many directions.

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THE PHYSIOLOGY OF SEA-URCHIN SPERMATOZOA

THE DILUTION EFFECT IN RELATION TO COPPER AND ZINC

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(*Received* 14 *September* 1949)

(With Six Text-figures)

INTRODUCTION

The O_2 uptake of sea-urchin spermatozoa varies according to the density of the suspension, the rate per spermatozoon in dilute suspensions being higher than in more dense ones. Gray (1928*a*, *b*), who first observed this phenomenon, concluded that this difference was due to spermatozoa in dilute suspensions having more space to move, and therefore being able to expend more energy than those in dense suspensions. He called the inhibitory action of close-packing allelostasis; dilution may therefore be considered as an an-allelostatic stimulus, if Gray's hypothesis is correct. Apart from the increased rate of O_2 uptake associated with dilution, the total O_2 consumed per spermatozoon is higher in dilute than in dense suspensions. The possible causes of these phenomena have recently been examined in detail (Rothschild, 1948); only the conclusions need therefore be mentioned here. The following possibilities were considered:

(1) *Extracellular auto-intoxication.* Excluded.

(2) *Exhaustion of intracellular substrate.* Excluded.

(3) *Intracellular auto-intoxication.* Probably excluded.

(4) *Allelostasis.* Not a complete explanation.

(5) *Exhaustion of link in metabolic cycle.* Suggested, but no experimental data produced. Experiments have now been done.

(6) CO_2 *narcosis.* Excluded.

(7) *Inadequate* O_2 *saturation in dense suspensions.* Partly responsible. Further experiments are recorded in this paper.

In this paper the words 'very dense', 'dense' and 'dilute' sperm suspensions are defined as follows:

very dense	$d \geqslant 5 \times 10^9$ sperm/ml. (d = density)
dense	$4 \times 10^8 < d \leqslant 10^9$ sperm/ml.
dilute	$d \leqslant 4 \times 10^8$ sperm/ml.

MATERIAL

Semen of *Echinus esculentus* diluted with sea water (i.e. 'unwashed spermatozoa'). The device shown in Fig. 1 has been found convenient for the collection of sea-urchin semen. The testes are placed on top of the perforated Perspex disk in the glass funnel, and each is cut in half. However carefully the testes are dried with filter-paper beforehand, a certain amount of watery fluid at first drops into the beaker below the funnel. This is discarded and the semen proper is collected through bolting silk.

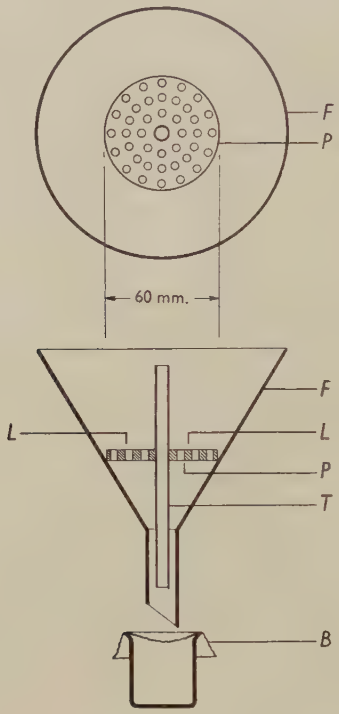

Fig. 1. Device for collecting sea-urchin semen. *B*, bolting silk; *F*, funnel; *L*, location of testes; *P*, perforated Perspex disk; *T*, glass tube.

METHOD

Manometric procedure. O_2 uptake was measured in Warburg manometers, $k \doteqdot 1{\cdot}2$, fitted with conical vessels of about 13–15 ml. capacity. Except in certain experiments specifically mentioned, the vessels contained 2·4 ml. of sperm suspension and 0·2 ml. 10% KOH in the centre cup, which also contained a roll of filter-paper (Whatmann, no. 40) protruding 0·5 cm. over the edge of the cup and 'flowered' to increase the absorbent surface. Reagents were added from side-arms. The gas space contained air.

Experiments were done at 15° C. Unless otherwise stated, the shaking rate was 100 c.p.m. with a 3·5 stroke. The equilibration time was 15 min.

Sperm counts. Photoelectric absorptiometer (Rothschild, 1950*a*).

RESULTS

Dilution Effect. Gray's experiments were repeated under rigorous and quantitative conditions. The principal result, that sea-urchin spermatozoa respire at a higher rate in dilute than in dense suspension, was confirmed. Sea water (1·9 ml.) was put in the manometer vessels and different amounts of slightly diluted semen (1:5) in the side-arms. At $t=0$, the contents of the side-arms were transferred quantitatively into the main vessels. By this method, O_2 uptake could be followed from the beginning of the experiment. The results are shown in Fig. 2. The concentrations of spermatozoa in each vessel were: curve I, 5.58×10^8/ml.; curve II, $8{\cdot}00 \times 10^8$/ml.; curve III, $1{\cdot}02 \times 10^9$/ml.; curve IV, $1{\cdot}22 \times 10^9$/ml. The sperm concentrations are deliberately given per ml.; to give the *total* number of spermatozoa

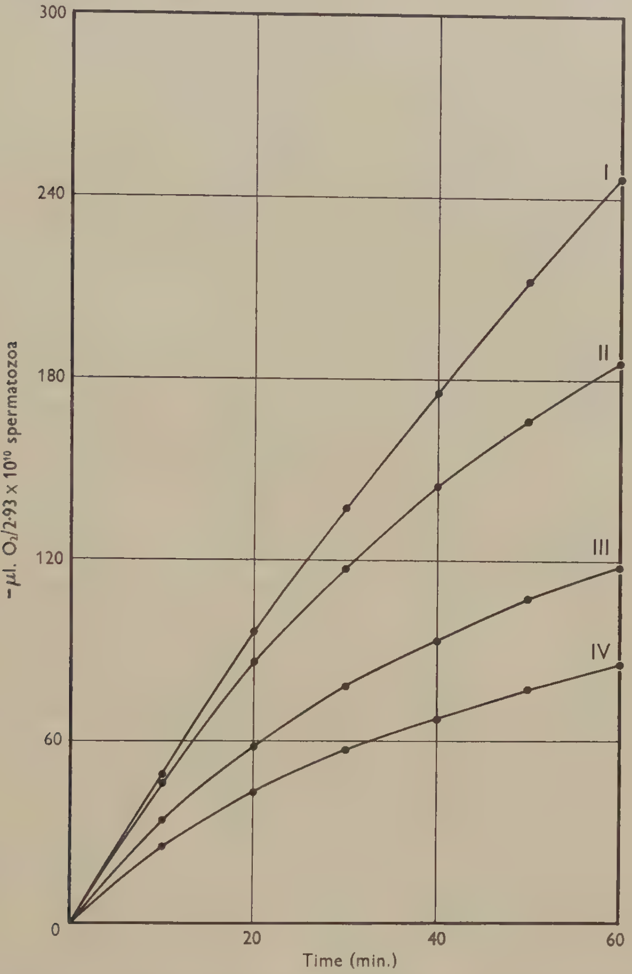

Fig. 2. Dilution Effect. O_2 uptake per unit quantity of unwashed sperm at different sperm densities. Number of sperm per ml.: I, $5{\cdot}58 \times 10^8$; II, $8{\cdot}00 \times 10^8$; III, $1{\cdot}02 \times 10^9$; IV, $1{\cdot}22 \times 10^9$.

per vessel would be meaningless unless the total volume, which varies in each case, were given at the same time. The total number of spermatozoa in each vessel can be obtained by multiplying the figures given above by the factors 2·4, 2·3, 2·2 and 2·1, which are also the ml. of sperm suspension in each vessel. The original concentration of spermatozoa in the undiluted semen was $2{\cdot}93 \times 10^{10}$/ml., and all four curves have been adjusted according to the number of spermatozoa in the relevant manometer, so that each records the O_2 uptake per unit quantity of spermatozoa, at the dilution in question. There are two points to note in Fig. 2. First, the existence of the Dilution Effect is confirmed. Secondly, the O_2 uptake declines more rapidly in the dense than in the dilute suspensions. The significance of this observation is discussed later, though mention should be made here that it has been shown (Rothschild, 1948) that no harmful or inhibitory substances diffuse out of dense sperm suspensions into the external medium.

Inadequate O_2 *saturation in dense suspensions.* In an earlier paper (Rothschild, 1948), it was shown by the technique of varying the shaker speed that O_2 saturation was probably a limiting factor in the O_2 uptake of dense suspensions of sea-urchin spermatozoa. Although the O_2 uptake of such suspensions could be raised to a certain extent by increasing the O_2 tension in the gas space in the manometers, the increase was quite insufficient to account for the Dilution Effect. If, however, very dense suspensions, containing for example 5×10^9 sperm/ml., are used, O_2 saturation severely limits respiration. The previous experiments on O_2 saturation have been confirmed, using a more delicate, but more difficult technique, that of comparing the O_2 uptake, per unit quantity of sperm, of different amounts of the same sperm suspension. This means that each manometer vessel contains a different thickness of the same sperm suspension. From these experiments it can be concluded that not more than about 10% of the difference between the O_2 uptake of dense and dilute suspensions can be attributed to inadequate O_2 saturation, unless undesirably high sperm suspensions are used. In the latter case, very high shaking speeds, which damage the spermatozoa, are necessary. Alternatively, measurements must be made on such thin layers of suspension that other difficulties are introduced.

Effect of $CuCl_2$. The addition of $CuCl_2$ to a suspension has a profound effect on O_2 uptake (Fig. 3). In this experiment, the O_2 uptake of the suspension exhibited a characteristic decline with time (cf. Gray, 1931). After 2 hr., $CuCl_2$ in isotonic NaCl was added to the suspension from the side-arm, the final concentration being 25 p.p.m. (parts per million).* There is an extraordinary and immediate increase in O_2 uptake. The stimulating action of $CuCl_2$ is proportional, over certain limits, to the amount added (Fig. 4). In this experiment the final concentrations of $CuCl_2$ were: curve I, 25 p.p.m.; curve II, 5 p.p.m.; curve III, 1 p.p.m.; curve IV, zero.

Effect of isotonic NaCl. In the above experiments the copper solutions in the side-arms were made up in 'Analar' NaCl, isotonic with sea water. The same applies in all experiments unless the solvent is specifically mentioned. 'Analar' NaCl was selected as the solvent for an important reason, which affects the interpretation of nearly all the experiments described in this paper. The reason and its implications are:

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* The significance of the word 'concentration' as applied to copper salts in sea water is considered in the Discussion Section; 1 p.p.m. $CuCl_2$ is equivalent to a $7{\cdot}44 \times 10^{-6}$ M solution.

(1) The addition of 0·28 ml. isotonic 'Analar' NaCl to 2 ml. sperm suspension, density 10^9/ml., never caused any increase in O_2 uptake. 'Analar' NaCl contained negligible quantities of Cu or Zn.

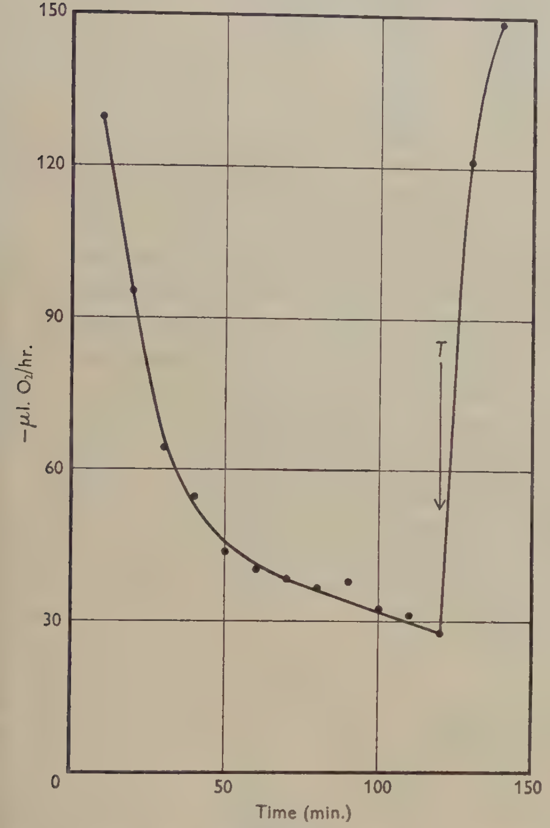

Fig. 3. Effect of adding $CuCl_2$, final concentration $1·86 \times 10^{-4}$ M (25 p.p.m.) at time *T*, on O_2 uptake of unwashed sea-urchin spermatozoa. Sperm concentration, approx. 10^9/ml.

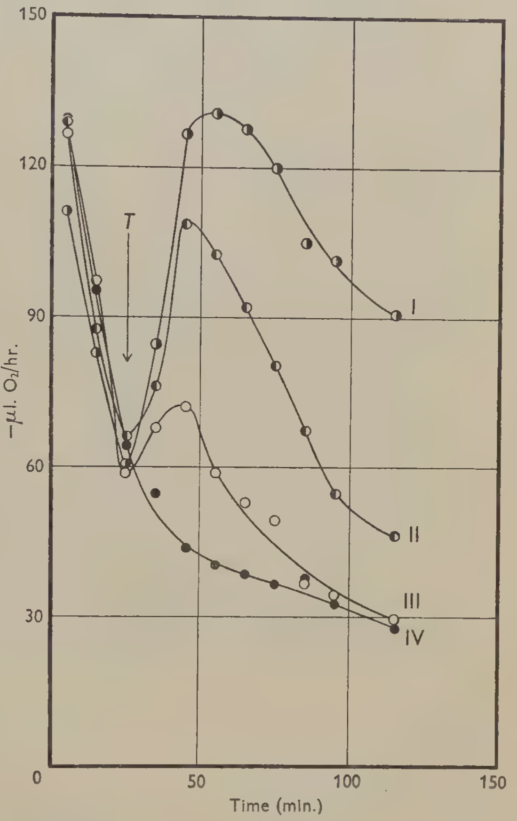

Fig. 4. Effect of adding different amounts of $CuCl_2$ at time *T*, on O_2 uptake of unwashed sea-urchin spermatozoa. Final concentrations of $CuCl_2$: I, 25 p.p.m.; II, 5 p.p.m.; III, 1 p.p.m.; IV, 0. Sperm concentration, approx. 10^9/ml.

(2) A dilution of 1·14 at this sperm density is therefore insufficient to cause a measurable Dilution Effect. The dilution of the sea water following the addition of this amount of isotonic NaCl is too slight to have any harmful effect.

(3) Suppose that with 2 ml. of 10^9/ml. sperm suspension in the main vessel, a shaking rate of 100 c.p.m. and a 3·5 stroke, there is inadequate O_2 saturation. It might be that when the $CuCl_2$ solution is tipped into the main vessel, the resultant agitation of the sperm suspension causes a temporary improvement in O_2 saturation, with a concomitant increase in O_2 uptake. There are several reasons for rejecting this hypothesis (see Discussion Section). One is that if it were correct,

the addition of isotonic 'Analar' NaCl, like $CuCl_2$ in isotonic 'Analar' NaCl, should cause an increase in O_2 uptake: it does not.

Sea water was not used as a solvent for the copper solutions in the side-arms because of uncertainties regarding its Cu and Zn content (cf. Glaser & Anslow, 1949).

During the experiments on the addition of isotonic NaCl to sperm suspensions, an opportunity arose to compare the effects on O_2 uptake of isotonic solutions of 'Analar' NaCl and 'Bench' NaCl. The first of these had no influence. It contained negligible quantities of Cu and Zn. The second caused a marked increase in O_2 uptake; spectroscopic examination, for which we are much indebted to Dr R. L. Mitchell, the Macaulay Institute for Soil Research, Aberdeen, revealed that 'Bench' NaCl contained approx. 7 p.p.m. Zn and 1 p.p.m. Cu. The samples contained no Mn and negligible quantities of Co, Ni, Ti, V, or Cr.

Effect of $CuCl_2$ *on* O_2 *uptake of suspensions containing different numbers of spermatozoa.* If Cu is a factor in sea-urchin sperm metabolism and is present in marginal quantities, dense suspensions might use any externally available Cu more quickly than dilute suspensions. If this were so, dilute suspensions, whose O_2 uptake does not decline so quickly as that of dense suspensions, should be relatively unaffected by the addition of Cu. The results of an experiment to test this hypothesis are shown in Table 1. The addition of $CuCl_2$ markedly restored the level of O_2 consumption in the dense suspension, but had no effect on the more dilute suspension. The table also shows that the O_2 uptake per unit quantity of spermatozoa is much lower in the dense suspension, for the values under V_1 must be divided by 2·5 (figures in brackets) for quantitative comparison with the values under V_2. This is the Dilution Effect. Secondly, before the addition of $CuCl_2$, the rate of respiration falls four times as much in the dense as in the dilute suspension.

Table 1. O_2 *uptake of sea-urchin spermatozoa*, Echinus esculentus

$T°$ C., 15. Shaker, 100 c.p.m.

Vessel no.	V_1		V_2	
Main vessel	2×10^9 sperm in 2 ml. suspension KOH, filter-papers		4×10^8 sperm in 2 ml. suspension KOH, filter-papers	
Side-arm	$CuCl_2$, 5 p.p.m.		$CuCl_2$, 5 p.p.m.	
Gas space	Air		Air	
Time (min.)	$-\mu l.\ O_2$	% of original rate	$-\mu l.\ O_2$	% of original rate
0–20	29 (12)		25	
20–40 TIP	19 (8)	−35	23	− 8
40–60	29·5 (12)	+ 2	23	0
60–80	30·5 (12)	+ 5	21	−16

Comparison of $CuCl_2$, $CuCl$, $CuSO_4$ *and egg-water.* The results of an experiment in which these substances were compared is shown in Fig. 5. Reference has already been made to the use of the word 'concentration' in regard to Cu compounds in sea water, and the point is discussed in detail later. The difficulty applies even more in the case of CuCl than $CuCl_2$. In this experiment there were four manometers. Each vessel contained 2·0 ml. sperm suspension, density approx. 10^9/ml. The side-arms contained 0·28 ml. of: curve I, 20 p.p.m. CuCl; curve II, 50 p.p.m. $CuCl_2$; curve III, egg-water; curve IV, 50 p.p.m. $CuSO_4$. The final concentrations of Cu salts therefore were: I, 2·5 p.p.m. CuCl; II, 6 p.p.m. $CuCl_2$; IV, 6 p.p.m. $CuSO_4$. Fig. 5 suggests that on the basis of the alleged concentrations CuCl is more efficient in reversing the decline in O_2 uptake than $CuCl_2$. $CuSO_4$ was less efficacious than CuCl or $CuCl_2$, while egg-water occupied an intermediate position. It will be noted

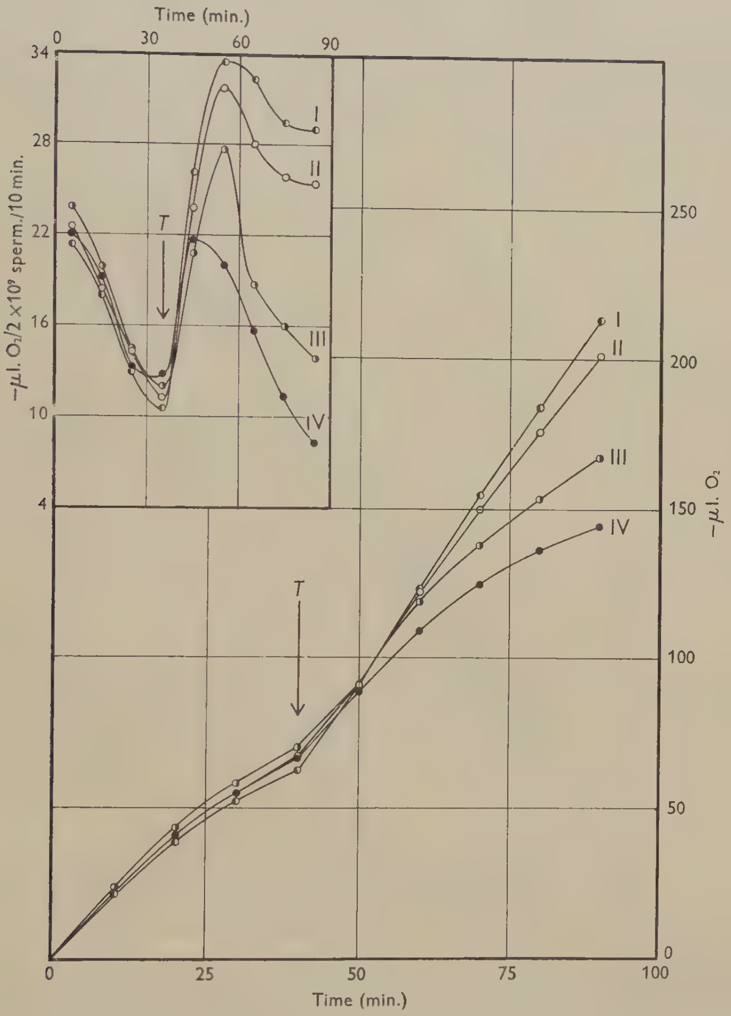

Fig. 5. Effect on O_2 uptake of unwashed sea-urchin spermatozoa of CuCl, $CuCl_2$, egg-water and $CuSO_4$. Final concentrations: I, 2·5 p.p.m. CuCl; II, 6 p.p.m. $CuCl_2$; III, egg-water; IV, 6 p.p.m. $CuSO_4$. Sperm concentration, approx. 10^9/ml.

that CuCl and $CuCl_2$ do not cause a transient increase in O_2 uptake; the increase is maintained.

O_2 *uptake in the presence of sodium diethyldithiocarbamate* (DEDTC) *alone and in conjunction with* $CuCl_2$. Fig. 6 shows the effects of DEDTC and DEDTC + $CuCl_2$. Each of the four manometer vessels contained 2 ml. sperm suspension, density approx. 10^9/ml. The side-arms contained DEDTC in sea water, curve IV; DEDTC + $CuCl_2$ in sea water, curve I; $CuCl_2$ in sea water, curve II; and sea water, curve III. After tipping, at $t = 40$, the concentrations of DEDTC and $CuCl_2$ in the main vessels were: I, DEDTC, $6{\cdot}24 \times 10^{-6}$M, $CuCl_2$, 0·6 p.p.m.; II, $CuCl_2$, 0·6 p.p.m.; III, control; IV, DEDTC, $1{\cdot}25 \times 10^{-5}$M. DEDTC, which inhibits reactions in which Cu is involved, has a pronounced inhibitory action on the O_2 uptake of sea-urchin spermatozoa. Any inhibition there might have been at lower concentrations was nullified by the addition of $CuCl_2$. This point is further illustrated in Table 2. In this experiment, the manometer vessels each contained 2 ml. of sperm suspension, concentration approx. 10^9/ml., KOH, and filter-papers. The side-arms contained various amounts of $CuCl_2$ and DEDTC in sea water, which were tipped into the

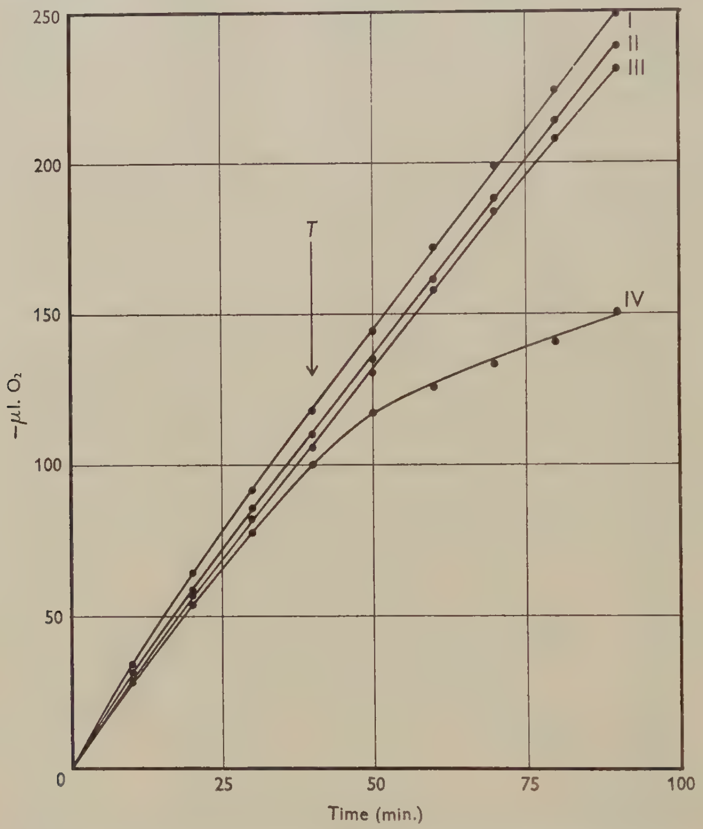

Fig. 6. Effect on O_2 uptake of unwashed sea-urchin spermatozoa of Diethyldithiocarbamate (DEDTC), $CuCl_2$, and DEDTC + $CuCl_2$ added at time *T*. I, DEDTC + $CuCl_2$; II, $CuCl_2$; III, control; IV, DEDTC. Sperm concentration, approx. 10^9/ml.

suspensions at $t = 0$. The final concentrations are given in the table. The gas space contained air. When examining this table it is important to compare the differences in O_2 uptake in the periods 0–10 and 10–20 in each column, and not to make lateral comparisons, because the stimulating effect of $CuCl_2$ tends to take place more quickly than the inhibitory action of DEDTC. Moreover, there is a considerable scatter, due in part to pipetting errors, between the readings in different vessels. Table 2 shows that DEDTC alone inhibits O_2 uptake (V_1), but that if sufficient Cu is added at the same time even when the concentration of DEDTC is relatively high, this inhibition is reduced or nullified (V_2). Conversely, the stimulating action of Cu is reduced by DEDTC (V_4).

Table 2. O_2 *uptake of sea-urchin spermatozoa,* Echinus esculentus

T° C., 15. Shaker, 100 c.p.m.

Vessel no.	V_1	V_2	V_3	V_4	V_5
Final concentrations of					
$CuCl_2$, p.p.m.	0	1·23	1·23	0·12	0
DEDTC, $M \times 10^{-6}$	6·14	12·28	0	6·14	0
Time (min.)	$-\mu l.\ O_2/hr./10^9$ sperm				
0–10	68	96	96	83	97
10–20	31 (−54)	88 (−8)	86 (−9·6)	44 (−47)	80 (−18)
20–30	33	93	96	45	74

The figures in brackets are the percentage differences between the readings for the periods 0–10 and 10–20.

Influence of other metals on O_2 *uptake of spermatozoa.* $ZnCl_2$ has a similar stimulating effect on respiration as $CuCl_2$. The increased O_2 uptake following the addition of small quantities of isotonic 'Bench' NaCl may therefore have been due to Zn, as the final concentration of Zn was about 1 p.p.m. while that of Cu was about 0·1 p.p.m. Mn and Fe did not stimulate O_2 uptake.

DISCUSSION

O_2 *saturation of sperm suspensions.* Before discussing the Dilution Effect, some attention must be given to the O_2 saturation of sperm suspensions in experiments using Warburg manometers. Although there is no difficulty in verifying experimentally that O_2 saturation is not a limiting factor in a manometric experiment, by altering the shaking rate, or having different thicknesses of the same suspension in a series of manometers, the possibility of inadequate O_2 saturation must always be borne in mind in experiments involving high concentrations of sperm. To take an extreme case, it would be impossible to measure the O_2 uptake, if any, of undiluted sea-urchin semen in Warburg manometers, which normally require 1–2 ml. of biological material in the main vessel. If undiluted semen is put in a Warburg manometer and shaken at 100 c.p.m., it is easy to see that the semen is hardly agitated at all. All the spermatozoa, except those in the uppermost surface layer, are in a condition of permanent anoxia, in which no movement takes place. When investigating any aspect of sea-urchin sperm metabolism in Warburg manometers, the maximum permissible sperm concentration is about 10^9/ml. or 1/20. Even at this concentration there may be a small O_2 deficiency effect unless high shaking speeds are used. Provided that the sperm suspension is not too dense, a reduction in O_2 tension, say to 20–40 mm. Hg, does not cause any O_2 deficiency effect because low O_2 tensions have no influence on sperm O_2 uptake (Rothschild, 1948). O_2 deficiency means that a significant number of spermatozoa are not respiring at all, because there is no O_2 available, or are respiring at a reduced rate because the O_2 tension is only a fraction of a mm. Hg.

Decline in O_2 *uptake of a sperm suspension.* In this section we shall develop the argument that there are two forms of decline in O_2 uptake: one of these occurs in dilute suspensions ($\leqslant 1/50$) and no way of reversing it has yet been found, though it can be retarded; the other occurs in dense suspensions ($1/50 < d \leqslant 1/20$) and can be reversed by the addition of Cu and Zn salts. In very dense suspensions (1/4), the addition of sea water will decrease the viscosity of the suspension and agitate it, with the result that O_2 saturation will at any rate temporarily improve. Apart, therefore, from receiving an an-allelostatic stimulus, the suspension consumes more O_2 because it obtains more of the O_2 it requires. The Dilution Effect occurs, however, when the sperm density in the suspension is such that inadequacy of O_2 saturation is not an important factor. It does *not* occur when isotonic 'Analar' NaCl solution is added, but does when Cu or Zn is present in the NaCl solution. Moreover, the increase in O_2 uptake is proportional, within certain limits, to the amount of Cu added, which would naturally not be the case if the addition of NaCl solution containing small quantities of Cu were only an an-allelostatic stimulus. Finally, diethyldithiocarbamate, which inhibits reactions in which Cu is involved, reduces the O_2 uptake of the suspensions, which suggests that Cu is concerned in the metabolism of sea-urchin semen. The experiments do not completely obviate the possibility that DEDTC has a narcotic effect, but this interpretation of the results seems unlikely to be correct.

The effect on O_2 uptake of adding sea water to a sperm suspension probably depends both on the density of the suspension and on the composition of the sea water at the time of the experiment, which we believe, as do Glaser & Anslow (1949), may vary according to the organisms which have previously been in it, or even with physical factors such as illumination. Pronounced increases in O_2 uptake following addition of sea water only occur when dense suspensions, 10^9/ml., are diluted. Unlike Cu in such cases, sea water is variable in its action and it is reasonable to assume that this variability is due to the sea water rather than the spermatozoa, which always react in the same way to a diluent of known composition, i.e. NaCl solution containing Cu, or NaCl solution containing no Cu or other impurities. These observations lead one to suspect that sea water may contain small quantities of some element which has a role in the metabolism of the sea-urchin semen. The fact that added Cu has less effect on dilute suspensions, though in these it often arrests the decline in O_2 uptake, might be taken as confirmatory evidence for the hypothesis that the Dilution Effect with sea water is not caused by the sea water as a whole, but by the Cu in it, though the amounts normally available must be extremely marginal from the point of view of the spermatozoa if there is nearly enough for a suspension of density 4×10^8/ml., but not enough for one with a density of 10^9/ml.

Cu *in sea water.* The solubility relations of Cu in sea water are complex. At pH 8 the theoretical concentration of Cu in sea water is about $1/1{\cdot}7 \times 10^7$, but this is much less than the values found for the solubility of the substance described as 'basic copper carbonate' (Barnes & Stanbury, 1947). Possibly Cu may remain in suspension in sea water for a considerable time as colloidal particles composed of insoluble basic complex salts. At the time of submitting this paper for publication no data exist regarding the concentration of Cu in Millport sea water. According to Sverdrup, Johnson & Fleming (1942), the Cu content of sea water varies from 0·001 to 0·01 μg./ml. (0·001–0·01 p.p.m.). Whether such an amount should be considered marginal from the point of view of the spermatozoa or not can only be the subject of speculation. The estimation of Cu in sea water before each measurement of sperm O_2 uptake would be a desirable precaution when these experiments are continued. But a more convenient way of standardizing conditions might be to use artificial sea water made up from re-crystallized salts and treated in the usual ways to remove metallic impurities, although Barron, Gasvoda & Flood (1949) state that the O_2 uptake of sea-urchin spermatozoa is lower in artificial than in natural sea water. Even with these precautions there are serious difficulties in knowing the true concentrations of added Cu salts in the sea water. The spermatozoa may physically adsorb part of the available Cu and utilize a further part in their metabolism, while Cu may diffuse out of dead spermatozoa. Cu may even be adsorbed on the glass of the manometer vessels.

An alternative and perhaps more satisfactory way of continuing these experiments under controlled conditions would be to carry out all estimations in the presence of DEDTC, adding Cu when required.

The concentrations of $CuCl_2$ and CuCl given in the Results Section are therefore liable to be seriously in error, in the sense that the alleged final concentrations are certainly much too high. They are recorded so that the initial conditions in the experiments are clearly defined.

Cu *in metabolism.* There is a vast literature concerning Cu in animal and plant metabolism, which has been summarized by Dawson & Mallette (1945). At this stage it would be unprofitable to speculate in detail on the possible functions of Cu in the metabolism of sea-urchin sperm or semen, though the inhibitory action of DEDTC supports the view that Cu is involved in their normal metabolism. A possible role would be in the catalytic oxidation of ascorbic acid (Stotz, Harrer & King, 1937). The production of H_2O_2 when this reaction is catalysed by cupric ions (Dawson & Mallette, 1945) would not be injurious to sea-urchin spermatozoa as, unlike mammalian spermatozoa, they contain catalase (Rothschild, 1950*b*).

In the experiments described earlier on, no distinction was made between the metabolism of sea-urchin spermatozoa and sea-urchin semen, which consists of spermatozoa *and* seminal plasma. The seminal plasma of mammals contains several enzymes, and substances which might be utilized as seminal as opposed to sperm substrates (Mann, 1949). The same might be true in the case of sea-urchin semen. Before, therefore, any assertion is made that Cu or Zn stimulate the O_2 uptake of sea-urchin spermatozoa, it must be proved that these substances are not exerting their effects on the seminal plasma, which, in the case of unwashed semen in sea water, is an important component of the suspension. After sea-urchin semen has been washed with sea water to remove the seminal plasma, the O_2 uptake of the resultant sperm suspension is markedly lower than that of the same number of sperm in the same amount of sea water containing seminal plasma. The 'protective' effect of seminal plasma or artificial substitutes is very well known in the field of mammalian sperm physiology, and has been investigated to a small extent in sea-urchin spermatozoa (Metz, 1945; Wicklund, 1949). This may explain the low Qo_2's reported by Barron *et al.* (1949). Direct comparison of their and our figures is not possible because they do not give the number of sperm per manometer vessel. The dry-weight method of expressing results is not considered satisfactory in studies of sperm metabolism (Mann, 1949; Rothschild, 1950*a*).

Zinc. Only a few experiments on the addition of Zn to sperm suspensions were carried out, but they left no doubt that Zn, like Cu, markedly stimulates sperm respiration. Apart from being the metal in the prosthetic group of carbonic anhydrase and possibly being concerned with the action of uricase and insulin, about the existence of which in sea-urchin spermatozoa nothing is known, the role of Zn in cell metabolism is obscure. The concentration of Zn in sea water is even lower than that of Cu, being about 0·005 μg./ml. (Atkins, 1936). According to Webb (1937) there is no Zn in the coelomic fluid of *E. esculentus*, though it contains eighty times as much Cu as sea water.

Although inorganic Cu can catalyse the oxidation of a substance such as ascorbic acid, in seminal plasma or spermatozoa, a similar explanation of the stimulating action of Zn is difficult to justify.

Role of Cu *in fertilization.* No experiments on the role of Cu in the fertilization reaction were made in this series and so far as is known, the main information on the subject was obtained by F. R. Lillie (1921) in his classical researches on the inhibitory action of Cu on the fertilization reaction in *Arbacia*. Lillie concluded that the inhibition was caused by a reaction between the eggs rather than the spermatozoa and Cu. Reference has already been made to the possibility that the limits within which Cu is physiologically active (but not of course lethally active) may be surprisingly small. Examination of Lillie's results confirms this view, for no inhibition of fertilization occurred when the sea water contained 0·4 p.p.m. of added $CuCl_2$ and a sperm density of about 2×10^6/ml., while there was complete inhibition with the same sperm density and an added $CuCl_2$ concentration of 2 p.p.m., only five times more. Runnström (1939) has also examined this question.

Motility of spermatozoa. In this series of experiments, no attempt was made to make quantitative correlations between the stimulating action of Cu or Zn salts and the motility of the spermatozoa. The difficulties associated with estimates of motility are well known. Our view is that neither Cu nor Zn salts increase the motility of spermatozoa, though high concentrations of Cu have an inhibiting effect.

Purity of reagents. An inescapable conclusion to be drawn from these results is that little weight can be attached to experiments in which the O_2 uptake of unwashed sea-urchin sperm suspensions is increased by the addition of substances such as echinochrome, alcohols, or alleged substrates, unless it can be shown that neither the substances themselves, nor the media in which they are made up, contain metallic impurities. In the case of Cu, which is of special importance, a method of obviating its stimulating action has been suggested earlier on; but even in this case, it is desirable to have some information about the Cu content of the sea water, distilled water, and reagents being used. These arguments will apply most strongly if subsequent experiments show that Cu and Zn stimulate the O_2 uptake of sea-urchin spermatozoa as opposed to sea-urchin semen suspensions.

SUMMARY

1. When a suspension of sea-urchin semen in sea water is diluted, the total O_2 consumed and the rate of O_2 uptake per spermatozoon are greater than before dilution. This is the Dilution Effect, first described by Gray (1928*a*). A further investigation of this phenomenon has been made, using the semen of *Echinus esculentus*.

2. The form of the O_2 uptake-time curve of such suspensions varies according to the ratio semen:sea water.

3. In very dense suspensions ($d \geqslant 5 \times 10^9$ sperm/ml.), the apparent low O_2 uptake per unit quantity of spermatozoa is mainly due to inadequate O_2 saturation of the lower layers of the suspension, in which the spermatozoa are virtually unable to respire. Such suspensions are not suitable for experiments in Warburg or Barcroft manometers.

4. In dense suspensions ($4 \times 10^8 < d \leqslant 10^9$ sperm/ml.) the Dilution Effect was observed when sea water was added to the suspension, in such proportions that the sperm density was only reduced by a factor of 1·14. No Dilution Effect occurred when isotonic 'Analar' NaCl was added in the same proportions. On the basis of the small dilution involved, and the negative results with 'Analar' NaCl, it is concluded that the Dilution Effect is not exclusively due to the sperm having more space to move after dilution and therefore being able to expend more energy.

5. The Dilution Effect occurs when isotonic 'Analar' NaCl containing 1 p.p.m. $CuCl_2$ is added to a dense sperm suspension, in the above proportions. Stimulation of O_2 uptake is proportional, over certain limits, to the amount of $CuCl_2$ added. Both CuCl, $ZnCl_2$, and to a lesser extent $CuSO_4$, have similar effects.

6. In a particular experiment, egg-water and Cu salts were about equally efficacious in increasing the O_2 uptake of a sperm suspension.

7. The characteristic decline in the O_2 uptake of dense suspensions can be partly reversed by the addition of Cu salts.

8. In dilute suspensions ($d \leqslant 4 \times 10^8$ sperm/ml.), there is no Dilution Effect when sea water or $CuCl_2$ are added in the above proportions. On the other hand, the decline in O_2 uptake can be partly arrested by the addition of Cu salts.

9. Diethyldithiocarbamate markedly inhibits the O_2 uptake of unwashed sea-urchin spermatozoa diluted with sea water. The inhibition does not take place in the presence of added $CuCl_2$. Cu may therefore have a catalytic function in the metabolism of sea-urchin semen.

10. The possibility that Cu lack is responsible foı the reduced O_2 uptake of spermatozoa in dense suspensions is considered.

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THE FUNCTION OF HAEMOGLOBIN IN *CHIRONOMUS PLUMOSUS* UNDER NATURAL CONDITIONS

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(With Six Text-figures)

INTRODUCTION

Haemoglobin is found in all vertebrate animals; in blood it serves as a transporter and in muscles as a temporary store of oxygen. But in the invertebrates its significance cannot be so clearly defined.

Haemoglobin is spasmodically distributed among the invertebrate phyla. Where it occurs it is situated in various tissues; it is found in blood and other body fluids either in solution or in corpuscles, it is present in muscle, in glands or in nervous tissue. Owing to these different sites and because of variability in physico-chemical properties of the pigment attempts to generalize on its functions (cf. Lindroth, Krüger) are unsatisfactory. It is evident that haemoglobin must play different roles in different animals. It may act: (*a*) as it does in mammals as an oxygen absorber and transporter at all environmental oxygen pressures; (*b*) as an absorber and transporter only at low oxygen pressures; (*c*) as a store of oxygen; (*d*) both as a transporter and a store, or, finally, (*e*) it may have no function.

In order to determine the function of haemoglobin in an invertebrate it is necessary to investigate not only the physico-chemical properties of the pigment and the oxygen consumption of the animal both in normal circumstances and deprived of a functional haemoglobin by means of carbon monoxide, but also the ecological conditions of the animal's normal life. Very few data regarding the latter are available for any haemoglobin-bearing species, yet it is only from such knowledge of their ecology and behaviour under natural conditions that the true significance of the pigment, as distinct from its chemical potentialities under artificial laboratory conditions, can be assessed. This paper is a laboratory study of the behaviour of the larvae of *Chironomus plumosus* L. under as natural conditions as possible, with a view to correlating their responses to environmental respiratory conditions with the known properties of their haemoglobin.

Lankester (1867) first recorded haemoglobin in *Chironomus* larvae, but apart from observing that these insects occur in situations where the oxygen supply is often small, refrained from attributing any specific function to the pigment. Miall & Hammond (1900) and Pause (1919) observed that certain chironomid species swim upwards into well-aerated water at night and suggested that haemoglobin may enable the larvae to form an oxygen store for subsequent use during oxygen shortage. Leitch (1916), however, showed that its storage capacity was in fact very limited and could not be of significance during long periods of oxygen lack. From studies on the metabolism of larvae under various conditions, Harnisch (1936) and Ewer (1942) concluded that in normal animals the haemoglobin is used in oxygen transport at low external oxygen pressures such as the larvae must frequently encounter in nature. Its high oxygen affinity (Leitch, 1916; Fox, 1945) also indicates such a function. Harnisch (1936) worked with *C. thummi* larvae which were repaying an oxygen debt after anaerobiosis, and concluded that when the level of their metabolism increased during this process the haemoglobin became functional at all oxygen pressures; he considered that the main significance of the pigment lay in increasing the recovery rate. In similar experiments, however, on *C. plumosus* larvae recovering from anaerobiosis, I failed to confirm the functional use of haemoglobin in well-aerated water (Walshe, 1947*b*), perhaps because the amount of oxygen debt repaid by my larvae was much smaller than that found by Harnisch. The experiments did, however, indicate that in the initial stages of recovery the larvae show a great amount of activity, during which the haemoglobin was used in oxygen transport.

Lindroth (1942) studied *Chironomus* larvae under more natural conditions by getting them to build U-tubes in mud between two glass plates. He observed that their respiratory ventilation of the tube was intermittent, and he reverted to the opinion that the haemoglobin functions as an oxygen store, by providing the animal with oxygen during the short pauses between ventilatory activity. His work was, however, very preliminary in nature, and his theory of haemoglobin as an oxygen store is unsubstantiated by any experimental evidence. The work described below is an extension of that begun by Lindroth, and consists of studies of the behaviour of larvae under various respiratory conditions, together with spectroscopic examinations of the haemoglobin in relation to the animals' behaviour, and the behaviour of larvae whose haemoglobin was made functionless by treatment with carbon monoxide.

METHODS

The larvae of *C. plumosus* live in U-shaped tubes in soft mud at the bottom of lakes and ponds. In the laboratory, larvae put into an aquarium containing several centimetres of sieved mud immediately burrow into it and they establish their tubes within a few hours. Such tubes vary somewhat in proportions; those of final instar larvae usually extend to a depth of 3–6 cm. in the mud and have an internal diameter of 2–3 mm. Their walls are consolidated by salivary secretion, the larva applying this by means of its mouthparts and hooked anterior proleg. The larval tubes once established within an aquarium seem to be permanent, and the larva pupates within its tube. Under normal conditions larvae never completely leave their tubes, although they occasionally project from them to gather food from the surface of the mud; they always hold on to the mouth of the tubes with the posterior prolegs and withdraw hastily when disturbed.

For a detailed observation of the behaviour of larvae in their tubes it was found to be impracticable to watch the larvae in the aquaria in which some of them had fortuitously built their tubes against the glass, since the galleries of the tubes were never entirely in view, and the larvae often hid in the obscured part. An attempt was made to persuade the larvae to build between two apposed glass plates, with mud in the interstitial space, as done by Lindroth (1942), but this also was unsuccessful, as under these circumstances the larvae only built very atypical structures. Finally the larvae were housed in artificial tubes. These were U-shaped, of glass tubing with an internal diameter of 2·5 mm. and external dimensions simulating those of natural tubes, fixed on small rectangular celluloid trays (Fig. 1). Each tray carried from two to five such tubes according to the size of the aquarium in which it was suspended. Into each tube a freshly collected final instar larva was introduced with a pipette. The tray was filled to the depth of about 5 mm. with sieved mud and lowered into an aquarium containing aerated lake water. This was then left for 24 hr., during which time the larvae lined the inner surface of their glass tubes with salivary secretion and particles of mud. Larvae so established showed no tendency to leave their tubes and thus could be observed over long periods of time. By means of outflow and inflow glass tubes resting on the tray the water in the aquarium could be changed without disturbing the larvae; the conditions above the tubes could therefore be controlled while at the same time the larvae were under as natural conditions as possible.

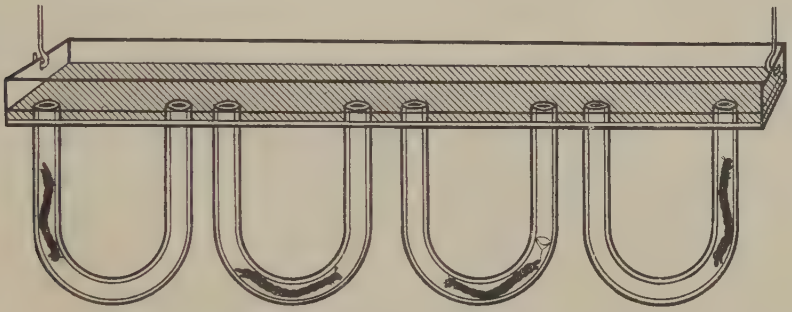

Fig. 1. Apparatus for observing the behaviour of *Chironomus plumosus* larvae. Description in text.

The behaviour of larvae was followed at a series of different oxygen and carbon dioxide pressures. Prior to observation the larvae were kept under constant lighting and respiratory conditions for 24 hr. They were then observed over a period of approximately 36 hr. Continuous observations of their behaviour for periods of either 30 or 60 min. were made at 3-hourly intervals. Undulations of the body were recorded on a kymograph by means of a tapping key, and other details of behaviour were noted. The oxygen content and pH of the water were frequently determined and the water changed when necessary. Oxygen determinations were made by the micro-Winkler technique (Fox & Wingfield, 1938) and the pH was estimated colorimetrically. The aquaria were kept at room temperature, which varied from 15 to 19° C.

In observing the behaviour of larvae with carboxyhaemoglobin the same technique was employed, except that before being placed in the desired experimental conditions the larvae were first left for 3 hr. in their tubes in aerated water containing dissolved carbon monoxide at a relative pressure one-sixth that of the oxygen in solution in the water. In this way the haemoglobin became converted into carboxyhaemoglobin, subsequent dissociation of which was prevented by maintaining a low (approx. 0·1–0·5 ml./l.) concentration of carbon monoxide in the aquarium water. The light intensity at which all the experiments were conducted was found to be insufficient to cause any dissociation of the carboxy-compound.

The state of oxygenation of the haemoglobin of individual larvae was studied by means of a hand spectroscope, the larvae being temporarily illuminated by a strong beam of light. The increased illumination necessary for the spectroscopic examination did not alter the behaviour of the larvae.

GENERAL BEHAVIOUR

(i) *Normal larvae*

The normal position of a larva within its tube is one in which the body is flexed so that the prolegs and the sensory setae on segment 12 are in contact with the tube walls. In this resting position the animal spends a large part of its life. Periodically, however, a series of rhythmic dorso-ventral undulations pass backwards along the body, driving water posteriorly. By renewing the water in the tube this irrigation* maintains the supply of dissolved oxygen to the larva, the animal regulating the time spent irrigating according to the oxygen and carbon dioxide pressures in the water; this intermittent irrigation of the tube may therefore be regarded as respiratory behaviour. From time to time, however, whatever their previous behaviour and irrespective of the external conditions, the larvae pass into a motionless phase which may last several hours. The immobility is complete; the larvae in a short time deplete the water in the tube of all oxygen and thus become subjected to anaerobic conditions. Such a motionless period can be artificially terminated by disturbing the larvae through vibration or sudden increase in light intensity, on which they irrigate their tubes. This sensitivity to disturbance is, however, only found after the animal has been motionless for some time.

The principal method of feeding in *C. plumosus* larvae is the filtering of planktonic organisms and particles from a stream of water passing through the tube. This filter-feeding mechanism has already been described in detail (Walshe, 1947*a*). Briefly, it consists in the larva spinning a cone of salivary secretion across the lumen of the tube, the apex of the cone sometimes being connected with the mouth by a thread; the larva then irrigates the burrow, the incoming water being drawn through the plankton net. The animal finally eats the net with its entrapped particles, and immediately spins another net. The entire performance takes less than 2 min., of which half is spent in irrigating the tube and half in eating and spinning the net, but it may be repeated many times, and the feeding sometimes continues for a number of hours. The whole forms a very stereotyped behaviour pattern which is easily recognized.

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* Most previous observers of this water movement in aquatic organisms have referred to it as a ventilation current. Since it is more correct etymologically, I am adopting the term irrigation current (Wells, 1945).

Although this is the commonest feeding mechanism, it is not the only one. On rare occasions I have seen larvae feeding from the surface mud. When doing this they extend the body out of the tube entrance, retaining contact with it by means of their posterior prolegs, and scrape the surface mud, at the same time spreading a net of salivary secretion over the surface, to which particles become attached and which is then dragged down into the seclusion of the tube for consumption. Occasionally larvae will also scrape the inner walls of their tubes with their mouthparts and anterior proleg, suggesting that they may also feed on the mud in which they live.

Finally, a very small part of the life of the larva is occupied by the consolidation of the walls with salivary secretion and by moving about and turning within the tube.

On the whole, however, the behaviour of a larva within its tube can be divided into three phases: filter feeding, immobility and intermittent irrigation. There seems to be no regular sequence in these various types of behaviour either under constant conditions or with diurnal light fluctuations, neither is the duration of any one phase constant under any set of conditions. Despite having larvae with identical histories for observation the behaviour of individual animals remains variable.

The general behaviour of larvae, however, was obviously influenced by the conditions of the water in which the larvae were kept. In well-aerated water they spend about 50% of their time showing respiratory behaviour, i.e. irrigating their tubes intermittently, the rest of the time being concerned with filter-feeding or remaining motionless (Table 1 and Fig. 2). Declining oxygen concentrations in the water have no effect on this general behaviour, although they alter the intermittency of the respiratory behaviour (see later), until they fall below 25% air saturation, below which there is a progressive decline in the time occupied by filter feeding and

Table 1. *Behaviour of larvae of* Chironomus plumosus *after 24 hr. under constant external conditions*

	Percentage air saturation of water	pH	No. of animals observed	Percentage total time		
				Filter feeding	Motionless	Respiratory behaviour
Normal animals	100–80	8·1	15	36	12	52
	37–22	8·1	15	32	16	52
	14–7	8·1	5	10	18	72
	7–5	8·1	4	0	12	88
	0	8·1	7	0	6	94
	0*	?	10	0	97	3
	76–70	6·5	5	16	3	81
	41–23	6·5	5	14	16	70
Animals with carboxyhaemoglobin	90–75	8·1	17	22	14	64
	39–26	8·1	19	11	46	43
	11–7	8·1	10	0	74	26

* After 5–6 days.

an increase in respiratory irrigation. A high carbon dioxide concentration (pH 6·5) has a similar effect. No filter-feeding was ever observed below 10% air saturation (0·7 ml. oxygen per litre at 18° C.); this is the most energetic process in the life of the larvae and it appears to be essentially an aerobic one. In completely oxygen-free water the behaviour of larvae was no longer constant over the observation period; at first, when the larvae had only been in anaerobic conditions for about 24 hr., the animals irrigated intermittently for most of the time. As the anaerobiosis continued, however, the amount of respiratory behaviour declined, the animals passing into a state of complete immobility. They were not, however, narcotized by the oxygen lack, for slight disturbance would cause them to irrigate for a few seconds before once again settling into a motionless state.

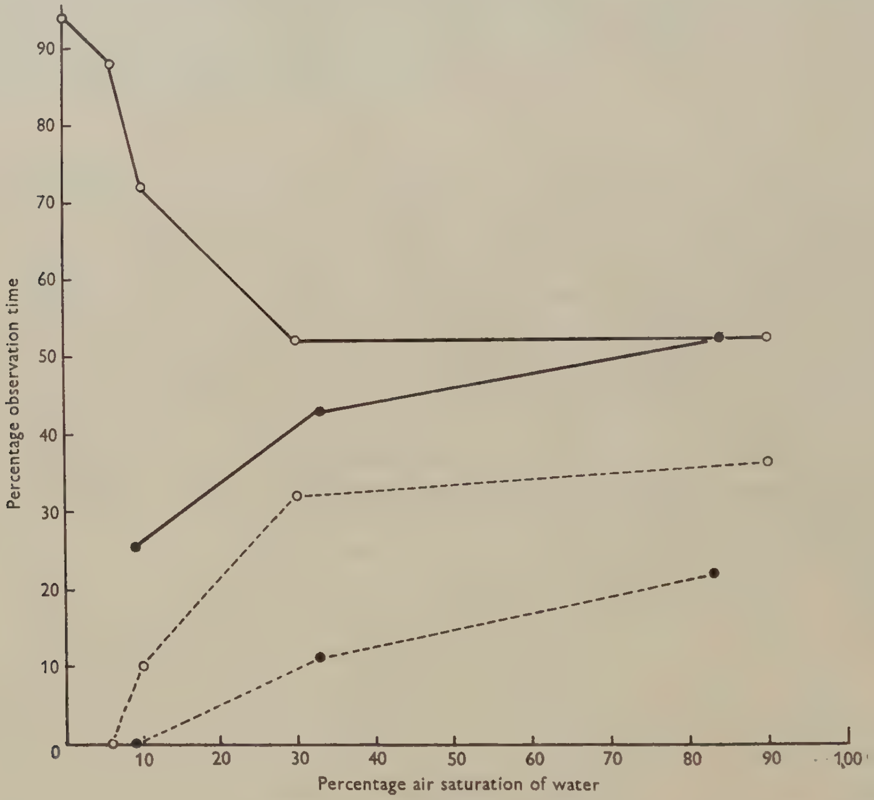

Fig. 2. Behaviour of *Chironomus plumosus* larvae at different oxygen concentrations, with and without functional haemoglobin. Data from Table 1. ○, normal animals; ●, animals with carboxy-haemoglobin. Unbroken line, respiratory behaviour; broken line, filter feeding.

The significant changes in behaviour of larvae during decline of oxygen in their surroundings is thus a reduction in the amount of filter-feeding with a corresponding increase in the time spent in the respiratory phase. As anaerobic conditions are approached respiratory irrigation of the tube is predominant until prolonged anaerobiosis results in immobility.

(ii) *Larvae with carboxyhaemoglobin*

Larvae without functional haemoglobin showed certain interesting differences in their responses to declining oxygen pressures (Table 1 and Fig. 2). In well-aerated water their behaviour is not strikingly different from that of normal larvae; they do, however, filter-feed rather less, and the filter-feeding pattern in several individuals was irregular and atypical, in that larvae would tend to spin odd salivary cones and then not carry out the irrigation part of the cycle. Reduction in the oxygen content of the water caused a rapid decline in the amount of filter-feeding which was not observed below 26% air saturation. Irrigation behaviour also decreased and the larvae consequently spent a large part of their time motionless; at 7% air saturation, for instance, the carbon monoxide-treated larvae were motionless for 74% of their time, while normal larvae showed a high degree of respiratory activity. The larvae with carboxyhaemoglobin, in fact, behaved as normal larvae do in anaerobic conditions.

The main effects of lack of functional haemoglobin are thus to prevent the larvae from filter-feeding and to reduce their level of activity, both these effects being greatest at low oxygen pressures. The inability to filter-feed at oxygen concentrations below 26% air saturation without haemoglobin indicates a new and interesting aspect of the function of the blood pigment; at low oxygen concentration it picks up and transports the necessary amount of oxygen for the high degree of secretory and muscular activity involved in filter-feeding. This must be of direct survival value since, although the larvae can feed by the less energetic process of eating from the walls of their tubes, larvae forced to do this by being kept in mud in plankton-free aquaria grow and metamorphose slower than those which have a plankton supply (Lang, 1931). Furthermore, the fact that at very low oxygen concentrations the carbon monoxide-treated larvae become immobile shows the value of haemoglobin to the normal animal in maintaining an internal oxygen pressure high enough to allow of respiratory activity and to ward off the immobilizing consequences of anaerobiosis.

RESPIRATORY BEHAVIOUR

(i) *Normal larvae*

So far, the general effects on the larvae of changing environmental conditions have been described. If one now considers only the respiratory behaviour, it can be seen that regulation of the lengths of irrigation periods and pauses occurs when the oxygen or carbon dioxide pressure of the water is changed.

Chironomus larvae irrigate their burrows intermittently; thus their oxygen supply, drawn from the incoming water during irrigation, ceases when the larvae pause. During the rest periods the oxyhaemoglobin and the small volume of tube water furnish a limited source of oxygen for the animals, but a prolonged pause will result in anaerobic conditions whatever the degree of oxygenation of the water above the tubes. If the chief functional value of the haemoglobin lies in forming a small store of oxygen for such short rest periods, the actual lengths of pauses between the periods of irrigation must be of significance. The irrigation during the respiratory

Table 2. *Analysis of irrigation behaviour of* Chironomus plumosus *larvae during the respiratory phase*

	pH 8·1					pH approx. 8·1	pH 6·5	
Average percentage air saturation of water	90	37	25	10	5	0	65	31
Percentage time irrigating during respiratory phase	2·5	9·4	11·8	59·1	88·0	17·6	16·7	31·3
Average length in min. of each irrigation period	1·5	3·9	4·9	>18	>30	8	2·7	>14
Average length in min. of each pause greater than 3 min.	11	13	13	10	0	8	6	8
Percentage of pauses less than 3 min. in duration	57	55	52	67	100	81	61	93

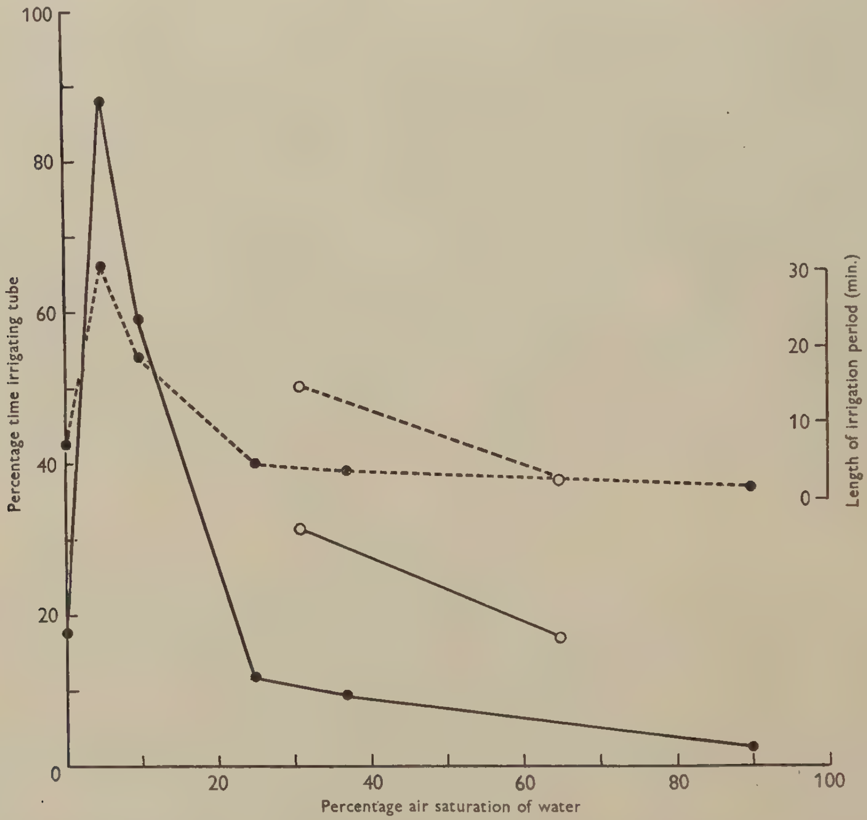

Fig. 3. Irrigation behaviour of respiratory phase larvae of *Chironomus plumosus* under various respiratory conditions. Data from Table 2. ○, data from experiments at pH 6·5; ●, data from experiments at pH 8·1. Unbroken line, percentage time irrigating tube; broken line, duration in minutes of each irrigation period.

phase has therefore been analysed by estimating the average lengths of the pauses and irrigation periods* under different conditions (Table 2 and Fig. 3). The kymograph records of the undulatory activity show that the intermittent irrigation tends to be very irregular. It became necessary therefore, in calculating the averages, to define the limits of a pause and of an irrigation period, for on the whole the irrigating periods did not consist of one well-defined period of continuous undulation, but rather of several bouts of activity separated by short pauses varying from a few seconds to one or two minutes in duration (Fig. 4). It was obvious that a truer picture of the animal's behaviour could be obtained by considering the grouped bouts of activity as all part of one larger period of irrigation, than by regarding each one separately. The lengths of the irrigation periods in Table 2 are the averages of such grouped periods of undulation, a period being considered as ended only when the larva remained still for three or more minutes. Thus at 10% air saturation few, and at 5% none, of the pauses are longer than 3 min.; consequently an upper limit to the lengths of the irrigation periods cannot be fixed. A true pause was considered as any still period greater than 3 min. and less than 30 min. in duration. If the larva remained motionless for more than 30 min. it was recorded as 'motionless' and not showing respiratory behaviour. This method of analysis is obviously arbitrary and artificial, since the lengths of pauses between irrigation periods cover the entire range from a few seconds to several hours, but some such treatment was necessary to present the behaviour numerically, especially as the durations of the long motionless pauses were largely unknown.

In well-aerated water a larva only spends 2·5% of its time irrigating the tube (Fig. 4*a*). Decline in the oxygen content alone (i.e. unaccompanied by an increased carbon dioxide pressure) has no effect on irrigation behaviour until it drops to between 50 and 45% air saturation; a preliminary series of experiments at 100, 80, 65, 60 and 45% saturation showed this to be the case. At 37 and 25% air saturation (Fig. 4*b*, *c*) the irrigation periods become progressively longer, although the pauses are still of the same duration. At the low oxygen concentration of 10% air saturation (Fig. 4*d*) the amount of irrigation is greatly increased and the pauses are now also reduced in length. At 5% air saturation (Fig. 4*e*), i.e. when only traces of oxygen are present, none of the rest periods is longer than 3 min.; the larva irrigates practically the whole time. This represents the extreme of respiratory regulation, for if during the recording of the behaviour the oxygen content sank to about 3% air saturation there was a noticeable decrease in irrigation and the larvae behaved as if in completely anaerobic conditions. In the latter (Fig. 4*f*) they show short outbursts of undulation which gradually decrease in frequency until they finally assume the immobility characteristic of prolonged anaerobiosis.

Although in this series of experiments the only variable condition was the oxygen content of the water, one cannot assume that the respiratory responses of the larva indicate a direct sensitivity to declining oxygen pressures. When the animal pauses between irrigation periods, simultaneously with the decrease in oxygen pressure there is a drop in pH owing to the production of carbon dioxide and other acid metabolic products. In fact, the changes in irrigation behaviour described above would indicate a sensitivity to pH changes rather than to oxygen deficiency itself, for there is no change in respiratory response over oxygen concentrations ranging from 100 down to 50% air saturation, yet since the metabolic rate is largely independent of the external oxygen concentration (Ewer, 1942) the tube water will be depleted of its oxygen much more rapidly at 50 than at 100% air saturation. Thus if irrigation were initiated by oxygen deficit alone one would find shorter pauses at 50 than at 100 % air saturation. Carbon dioxide, on the other hand, would be produced or released from buffering substances at roughly the same rate under both conditions.

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* The amount of oxygen available to a larva depends on the volume of water passing through its tube: the irrigation volume (a function of both the amplitude and duration of the undulatory activity) would therefore be a more satisfactory datum than the length of the irrigation period. For any individual animal, however, the amplitude and rate of beat remained constant under various conditions. Thus the length of the irrigation period alone gives an adequate representation of the irrigation of the tube.

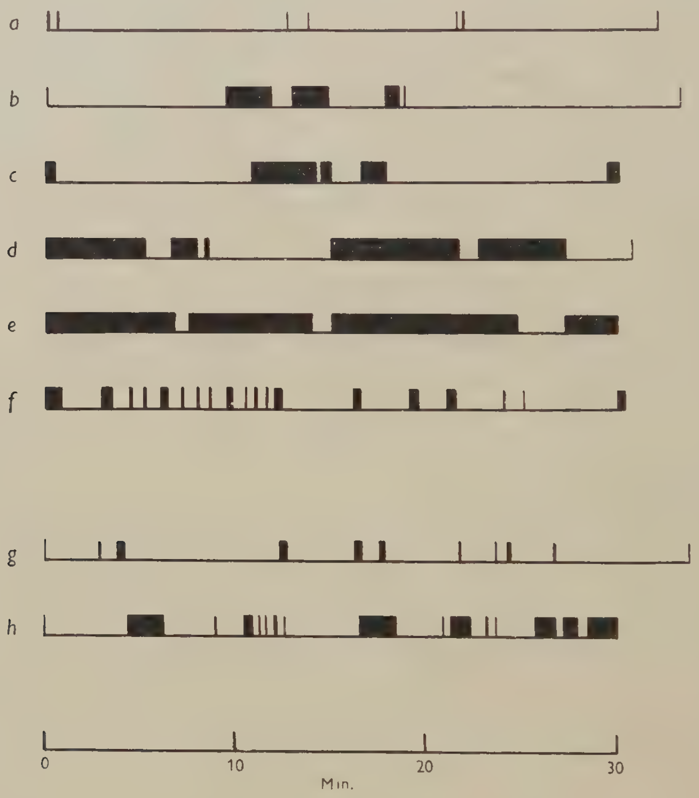

Fig. 4. Diagram based on kymograph records of irrigation behaviour of seven larvae of *Chironomus plumosus* under different respiratory conditions. Each line represents the behaviour of one individual over a period of approximately 30 min. Vertical lines or blocks indicate periods of irrigation of the tube, horizontal lines pauses when animal was resting. *a–f* at pH 8·1 and the following air saturations of the water: *a*, 90 %; *b*, 37 %; *c*, 25 %; *d*, 10 %; *e*, 5 %; *f*, 0 %. *g* and *h* at pH 6·5 and the following air saturations: *g*, 65 %; *h*, 31 %.

Thus a decreased pH within the animal's body probably initiates irrigation, and a return to the normal ends it.

The behaviour of larvae at a high carbon dioxide pressure (Fig. 4*g*) supports this interpretation. The carbon dioxide causes a lengthening of the irrigation period, as elimination of excess carbon dioxide by diffusion is slower, and a shortening of the pause as the threshold accumulation of acid within the body is reached sooner. At all oxygen concentrations, part of the animal's metabolism during a pause is anaerobic (see later). This implies the production not only of carbon dioxide but also of fatty acids, which, since there is no evidence for their continual accumulation as a large oxygen debt, must be eliminated during the subsequent irrigation period. Each irrigation period is, in fact, evidence of the elimination of a small oxygen debt. At 37 and 25% air saturation, although the pauses are as long as in more fully aerated water, the length of the irrigation periods is increased. This may well be due to a reduced rate of removal of the oxygen debt by oxidation of the anaerobic metabolites, at the lower oxygen pressures.

That the irrigation behaviour is, in fact, controlled by a factor such as changing pH and not by any internal nervous rhythm was shown by keeping larvae at a constant high oxygen pressure and pH. This was done by drawing well-aerated water through their tubes about every 20 min. with a fine pipette. Under these conditions the larvae assumed a state of apnoea and showed no spontaneous respiratory movements. Such larvae after an hour or two's treatment in this way no longer increased their amount of irrigation when placed in water containing little oxygen: the characteristic increased respiratory activity only became apparent after several hours. Conversely, animals previously kept at a fairly low oxygen concentration (e.g. 25% air saturation) immediately responded to a further decrease in oxygen. These data indicate that an animal showing respiratory behaviour has a certain basal accumulation of metabolic products within the body. Prolonged subjection to high oxygen concentrations removes this and causes a delay in respiratory responses. In other words, the animal has normally a permanent slight oxygen debt, above which additional accumulation of metabolites initiates irrigation. Elimination of these must become increasingly difficult at progressively lower oxygen concentrations until, finally, with poor incoming oxygen supplies the animal must be fighting a losing battle against anaerobic products. The decreased rate of irrigation and final immobility below 3% air saturation may be the result of their excessive accumulation.

Direct data as to how much anaerobiosis larvae endure at different oxygen concentrations, and what part the haemoglobin may play in reducing this were obtained by studying the haemoglobin in the living larvae with a spectroscope. With practice it was possible to detect various degrees of oxygenation of the haemoglobin and correlate these with the activity of the larva. Fig. 5 shows how gradual deoxygenation of the pigment occurs between bouts of irrigation when the animal remains still, and rapid reoxygenation takes place as the larva renews its water supply by irrigation, or occasionally by moving up to one of the ends of the tube. During the time when a larva remains still within its tube it has four possible sources of oxygen supply: (*a*) dissolved in physical solution in its blood, (*b*) contained in the oxyhaemoglobin, (*c*) obtained from the surrounding tube water by physical diffusion into the blood, and (*d*) absorbed from the tube water by the haemoglobin. Owing to its high oxygen affinity the blood pigment will only come into play after ready supplies of oxygen in physical solution have been exhausted and the tissue oxygen pressure has become sufficiently low to cause dissociation of the oxyhaemoglobin. The respiratory pigment will then be functional in two ways: by yielding its stored oxygen and by transporting the remaining small amounts of oxgyen from tube water to the tissues. As seen by the spectroscope, the time when the haemoglobin is thus functioning must be when the oxyhaemoglobin bands are progressively fading. Table 3 summarizes the spectroscopic observations. The blood pigment appears to be functioning most (i.e. is fluctuating between oxygenated and deoxygenated states for the greatest proportion of time) at the very lowest oxygen concentrations in the water (7·5–9 % air saturation). At 9 % air saturation of the water (0·6 ml. oxygen per l. at 18° C.) the haemoglobin remains partly oxygenated and at 7·5 % (0·5 ml. oxygen per l. at 18° C.) practically deoxygenated as long as the larva irrigates. Even though the oxygen demand of the tissues during this irrigation must be high, the pigment remains in a partly oxygenated state; this implies that as quickly as the animals' metabolism removes oxygen, it is picked up by the haemoglobin, which is thus functional in oxygen transport. At higher oxygen pressures the haemoglobin is functional over shorter periods of time.

The data on the amount of time during which the larvae have deoxygenated haemoglobin are of special interest (Table 3); these show that a considerable proportion of their time is spent under anaerobic conditions even when oxygen is available to them, and the amount of anaerobiosis endured is roughly constant at all oxygen pressures (the lowest value is at 7·5 % air saturation, under which conditions the metabolism must be largely anaerobic even when traces of oxyhaemoglobin are present). This constancy of amount of anaerobic metabolism lends support to the suggestion already advanced that respiratory behaviour is controlled by the accumulation of metabolic by-products during pauses between irrigation periods. In the experiments at pH 6·5 (Table 3) the increased carbon dioxide pressure caused such a rise in the amount of irrigation that the haemoglobin was fully oxygenated for most of the time and its periods of possible functioning considerably reduced.

Table 3. *The changes in oxygenation of the haemoglobin of* Chironomus plumosus *larvae in tubes at different oxygen concentrations of the water*

Percentage air saturation of water	pH	Number of animals	Percentage time irrigating	Percentage time with		
				Hb	$Hb \rightleftharpoons HbO_2$	HbO_2
100–75	8·1	8	3·0	41	38	21
75–60	8·1	4	3·3	53	16	31
45–30	8·1	4	9·5	36	22	42
30–15	8·1	8	14·9	55	24	21
15–10	8·1	6	50·0	28	27	45
9	8·1	2	61·0	38	63	0
7·5	8·1	2	80·0	20	80	0
100–75	6·5	2	55·0	4	8	88
30–15	6·5	4	85·0	4	2	94

The data so far discussed give no indication as to whether the storage or the transport function of the haemoglobin has the greater significance in the life of the larvae. The actual time in minutes for the larvae to deoxygenate their haemoglobin at different oxygen pressures is relevant in this connexion. In well-aerated water the haemoglobin is only completely depleted of oxygen after about 19 min., whereas at 15–30% air saturation of the water it becomes deoxygenated after 9 min. (Table 4, Fig. 5). Below 15% air saturation down to the oxygen concentration at which the haemoglobin is no longer fully oxygenated the deoxygenation time remains 9 min. At 10% air saturation the amount of oxygen available, and therefore transportable, in the small volume of tube water must be negligible, the 9 min. must therefore

Table 4. *The lengths of time for oxygenation and deoxygenation of the haemoglobin of* Chironomus plumosus *larvae in tubes at different oxygen concentrations of the water*

Percentage air saturation of water	Time for oxyhaemoglobin to become deoxygenated		Length of irrigation period (min.)	Time for haemoglobin to oxygenate	
	Normal animals	Inert animals		Normal animals	Inert animals
100–75	19	31	1·5	1·5	0·8
45–30	17	18	3·3	2·5	1·0
30–15	9	12	5·8	3·2	2·0
15–10	9	12	9·9	6·0	—

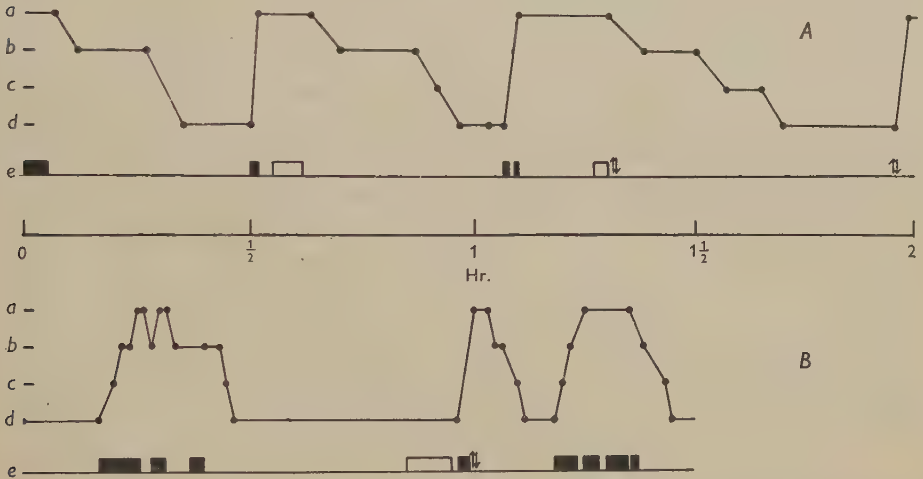

Fig. 5. The state of the haemoglobin of *Chironomus plumosus* larvae in tubes in relation to their behaviour. Parts of the records of two individual larvae: *A* in air-saturated water, *B* in 25% air saturated water. *a–d* are four recorded states of the haemoglobin: *a*, fully oxygenated, when absorption bands of oxyhaemoglobin at maximum intensity; *b*, partly oxygenated, when bands fading but still clearly double; *c*, almost deoxygenated when single absorption band of haemoglobin definite but with traces of double band of oxyhaemoglobin; *d*, deoxygenated when no trace of oxyhaemoglobin bands remained. Behaviour scale (*e*): straight line represents animal motionless within tube; solid blocks represent periods of irrigation; white blocks represent movement within tube; arrows indicate that animal moved to one of the ends of the tube.

represent the storage value of the haemoglobin to the normal animal. Thus at higher oxygen concentrations the extra time taken for deoxygenation shows that during the 17–19 min. the haemoglobin is also acting in the transport of oxygen from the tube water. These data were obtained from normal larvae which, in their respiratory regulation, become increasingly active as the oxygen concentration falls. If one now irrigates the tubes artificially with aerated water so as to produce a state of apnoea (see p. 83) before observation, the deoxygenation time is lengthened at all oxygen pressures; the metabolism is lower during the inactivity of the larva and the basal storage value becomes 12 min. (Table 4). This agrees exactly with the value arrived at by Leitch (1916), whose calculations were based on the metabolism of larvae free in water and therefore less active than irrigating animals; the storage value to an active larva under more natural conditions would seem to be about 9 min.

The reverse process of oxygenation of the haemoglobin during irrigation takes progressively longer as the oxygen falls (Table 4). In part this is due to decreasing rates of diffusion of oxygen through the cuticle, but not entirely so, since the haemoglobin of inert larvae artificially irrigated becomes oxygenated quicker than actively irrigating ones at the same oxygen pressure; the increased metabolism of the active animals draws on the oxygen supply of the reappearing oxyhaemoglobin and so lengthens the process of reoxygenation. Correspondingly the actual irrigation periods are longer.

These data indicate that the haemoglobin of the larvae showing respiratory behaviour may enable the animals not only to be more aerobic during their pauses but also to return to a high degree of aerobic activity more quickly when they are irrigating their tubes. To what extent the haemoglobin functioning in this way has actual significance in the life of the larva may be judged by a further analysis of the respiratory behaviour of larvae deprived of their haemoglobin by carbon monoxide.

(ii) *Larvae with carboxyhaemoglobin*

Returning to the experiments on larvae with carboxyhaemoglobin, Table 5 gives an analysis of their irrigation behaviour. The figures in brackets are the corresponding values for normal larvae to serve as a comparison. In aerated water the average results indicate that the larvae spend more time irrigating than normal owing to a decrease in length of pause and an increase in length of irrigation period. This is

Table 5. *Analysis of the irrigation of* Chironomus plumosus *larvae with carboxyhaemoglobin, during respiratory behaviour*

(pH 8·1. Figures in brackets are corresponding values for normal larvae)

Average percentage air saturation of water	90	30	10
Percentage total time respiratory behaviour	64 (52)	43 (52)	26 (72)
Percentage time irrigating during respiratory behaviour	5·6 (2·5)	11·6 (11·8)	35·3 (59·1)
Average length in min. of each irrigation period	2·5 (1·5)	2·2 (4·9)	8·5 (>18)
Average length in min. of each pause greater than 3 min. and less than 10 min.	8·5 (11)	9·1 (13)	7·6 (10)
Percentage of pauses < 3 min. in duration	71 (57)	57 (52)	72 (67)
No. of animals observed	15	13	4

what one would expect if the haemoglobin were functioning as suggested above. Examination of the individual records, however, show that these values are due to data from seven animals which were unusually active throughout the observation periods; their activity taking the form of scraping movements frequently interspersed with very short periods of irrigation. This behaviour was thought to be a feeding reaction; that it should be commoner in larvae with carboxyhaemoglobin may be related to their reduced amount of filter-feeding. The behaviour of the remaining eight animals was not noticeably different from normal, except for an increased tendency to move about within their tubes. This restlessness serves to mix the tube water and thus in normal animals to prolong the time during which they have oxyhaemoglobin. The lack of a functional haemoglobin may in this way be the cause of a slightly increased activity in the larvae, but in aerated water the effect is not a very obvious one.

In 30% air-saturated water also, the only outstanding difference in behaviour between animals with and without functional haemoglobin is the great total reduction in filter feeding in the latter. As regards respiratory behaviour alone there is no striking difference in irrigation between the two; the variability in behaviour makes the validity of the differences given in Table 5 doubtful.

At 10% air saturation only four out of the ten animals showed any respiratory behaviour, the rest remaining motionless throughout the observation periods. The behaviour of these four was of interest; two maintained a high rate of irrigation (78–88% of the time) such as is typical of normal animals at 5% air saturation, while the other two irrigated little and at frequent intervals their behaviour recalling that of normal animals after 24 hr. of completely anaerobic conditions. Finally, in their immobility, the six motionless individuals were reacting as do normal larvae under prolonged anaerobic conditions. Thus at 10% saturation all the larvae behaved as if the external oxygen pressure were lower than it actually was. At this oxygen pressure therefore the haemoglobin has a functional value in enabling larvae to maintain a high irrigation rate and so, thanks to its transporting properties to utilize the small amounts of available oxygen. Thus while carbon monoxide treatment has no very clearly defined effect on the respiratory behaviour of larvae in aerated water, at low oxygen pressures it causes the larvae to behave as if less oxygen were present than is actually the case.

RECOVERY FROM ANAEROBIOSIS

(i) *Normal larvae*

I have interpreted the intermittent irrigation of larvae showing respiratory behaviour as being initiated by the accumulation of metabolic end products and stopped by their removal after the resulting access to oxygen; each bout of irrigation is evidence of the elimination of a small oxygen debt. If animals are forced to be anaerobic for longer periods and so accumulate larger oxygen debts the subsequent irrigation behaviour is much more pronounced and the recovery slower. After any prolonged subjection to nitrogenated water the larvae, on returning to aerated water, irrigate strongly and continuously for many minutes, after which the amount of irrigation gradually sinks to normal. The speed of recovery depends on the oxygen concentration of the incoming aerated water; thus in a series of experiments in which larvae were kept for 4 days under anaerobic conditions and then transferred to various oxygen concentrations, the amount of irrigation returned to that typical of the particular oxygen concentration in less than 1 hr. in 75–100%, air saturation, 2 hr. in 45%, 2½–3 hr. in 10–28%, and 3 hr. in 7–22%. As the irrigation rate declines the activity of the larvae changes to filter-feeding until finally all individuals are feeding (Fig. 6). Below 7% air saturation larvae show no recovery; their irrigation rate remains high for several hours and there is no decline in rate associated with the adoption of filter-feeding. If left at this very low oxygen concentration they gradually resume anaerobic immobility.

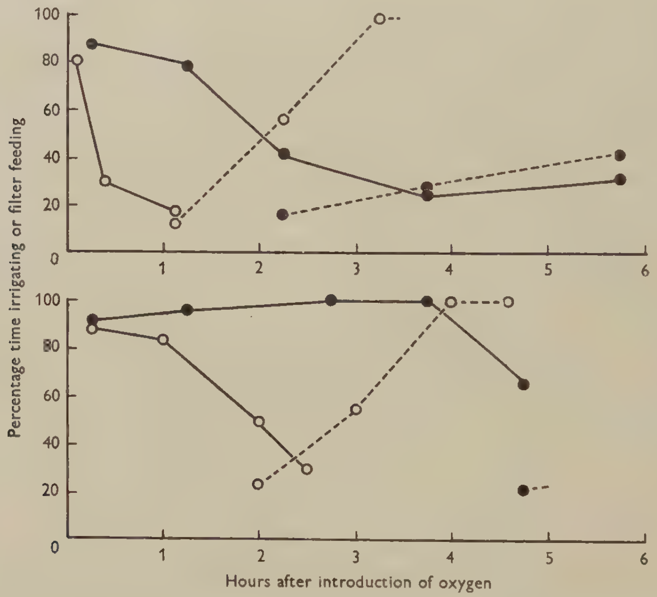

Fig. 6. Recovery of *Chironomus plumosus* larvae from a 96 hr. period of anaerobiosis: *A* in 67–100% air-saturated water; *B* in 10 rising to 28% air-saturated water. O, normal larvae; ●, larvae with carboxyhaemoglobin. Unbroken line, irrigation of tube; broken line, filter feeding.

(ii) *Larvae with carboxyhaemoglobin*

The recovery pattern of larvae with carboxyhaemoglobin is noticeably different (Fig. 6). In aerated water recovery, as judged by decline in amount of irrigation rate, is considerably retarded; indeed, even after 6 hr. it is higher than in normal animals after half an hour. Similarly, filter-feeding, almost always associated with recovery in normal larvae, is slower to appear. At lower oxygen concentrations the retarding effect of lack of haemoglobin is even more marked, the carbon monoxide larvae irrigating continuously for several hours. Below about 15% air saturation there is no recovery; as in normal larvae below 7% air saturation, the animals gradually return to an anaerobic existence. Without their haemoglobin, larvae would thus be at a considerable disadvantage when recovering from oxygen lack, especially if such recovery were taking place in water of low oxygen content.

The conclusions are in agreement with deductions based on a comparison of the metabolisms of larvae with and without haemoglobin when recovering from oxygen lack (Walshe, 1947*b*). In these experiments the animals were out of their tubes and free in slowly running water; nevertheless, they showed a high rate of undulation immediately after an anaerobic period, to maintain which aerobically the haemoglobin was functional in oxygen transport. After this initial outburst of activity the small oxygen debt (evident from an increased metabolism) was eliminated after an hour in fully aerated water, the same interval of time which larvae in their tubes need to return to normal behaviour. Carbon monoxide-treated larvae were also found to be able to repay an oxygen debt in aerated water, but the metabolism did not return to normal until about 2 hr. after the admission of oxygen: the larvae with carboxyhaemoglobin had actually a higher metabolism during recovery than normal animals, thus appearing to pay back a greater debt. The explanation of this was not clear at the time, but subsequently observation of the behaviour of carbon monoxide-treated larvae in their tubes suggested that it may well have been due to an undetected increased amount of irrigation activity during the lengthened recovery period. Thus, although larvae are capable of recovering from oxygen-lack in aerated water without the help of haemoglobin, the recovery time is considerably lessened by the possession of the blood pigment. Furthermore, the observation of the behaviour of larvae in tubes shows that lower oxygen concentrations recovery without the help of haemoglobin becomes increasingly difficult and finally impossible.

C. plumosus larvae are characteristic of bottom muds of highly productive stagnant waters in which oxygen depletion is a common phenomenon and completely anaerobic conditions not unusual. Re-aeration of the natural waters after periods of oxygen lack is bound to be slow, therefore the ability to reassume normal aerobic metabolism quickly and in spite of a low oxygen concentration must be of great significance in the life of the larvae.

Thus in post-anaerobic recovery prolonged vigorous irrigation of the tube occurs, and cessation of this and the adoption of filter-feeding marks the completion of recovery. Recovery is slower the less oxygen there is available, and larvae with carboxyhaemoglobin show a very prolonged recovery period, or at low oxygen pressures are unable to recover. Thus a functional haemoglobin is of significance in the resumption of normal metabolism after oxygen lack.

DISCUSSION

Under laboratory conditions intended to be as natural as possible the life of a *Chironomus* larva is seen to consist of alternating periods of activity and inactivity. Its most energetic behaviour is filter-feeding for which rapid secretion and undulation are maintained. Rest periods are very variable in length, ranging from short pauses of a few minutes' duration to long motionless periods of several hours. At the end of any pause, muscular irrigation activity serves to renew the animal's oxygen supply and if necessary to allow of repayment of any oxygen debt incurred during the previous pause. It remains now to assess the role of the animal's respiratory pigment during such behaviour.

The most recently advocated theory of the function of the haemoglobin of *Chironomus*, namely that it serves essentially as an oxygen store during short pauses (Lindroth, 1942, 1943), is based on Lindroth's observation of a regular periodicity in the respiratory movements of *C. plumosus* larvae; at 18–19° C. the animals irrigating for 5–10 min. and pausing for about 4 min. in well-aerated water. He supposed that during the pauses the oxyhaemoglobin maintained aerobic conditions and allowed the larvae to show other activities such as feeding. Lindroth's animals must have been much more active and regular in behaviour than mine, for he records no irregularities or long rest periods and inferred that the metabolism was essentially aerobic. In my animals, however, anaerobiosis was a normal phenomenon even with fully aerated water above the mud, for many of the pauses were of such a length that the storage value of the haemoglobin was inadequate to maintain aerobic conditions. Nevertheless, spectroscopic examination showed that the pigment does hold a 9 min. store of oxygen and this must reduce the degree of anaerobiosis to be endured by the resting animal. Were this of paramount importance in the animal's life, however, one would have expected the respiratory behaviour of animals without a functional haemoglobin to have been considerably different from normal, the removal of the oxygen store shortening the lengths of the pause. But such was not the case. With aerated water above the mud, larvae with carboxyhaemoglobin, although rather more irregular in their respiratory behaviour, showed no striking curtailment in the lengths of the pauses, and were if anything unusually active in lining their tubes and moving about during the intervals between irrigation periods. Larvae can therefore be active during pauses without the help of extra stored oxygen, and although the haemoglobin on account of its physico-chemical properties cannot help but be a short oxygen store during periods of inaccessibility of oxygen, its main significance in *Chironomus* does not appear to lie in this.

When the animals are lying free in water, the haemoglobin functions as an oxygen transporter at low oxygen concentrations (Ewer, 1942). In their natural tubes the oxygen pressure falls whenever the animals remain still: the conditions under which the haemoglobin can act as a transporter will therefore be present whenever the larvae pause. That the haemoglobin does in this way pick up oxygen from the tube water when the animals pause can be seen from the spectroscopic observations of the blood pigment inside the larvae in glass tubes. At oxygen concentrations above 30% air saturation the haemoglobin takes about 19 min. to lose all traces of oxygenation. Were it only giving up a store, deoxygenation would occur more rapidly (i.e. after 9 min. in an intermittently active animal). For some minutes therefore it is concerned with picking up oxygen from the tube water, and this together with its storage value postpones anaerobiosis. But the amount of oxygen in the tube water is small and the transporting ability of the haemoglobin during a pause is therefore limited.

The potentialities of the haemoglobin as an oxygen transporter during irrigation activity, when, because of the irrigation, the oxygen supply is unlimited, would seem to be of far greater significance. Thus at the lowest oxygen concentrations at which the haemoglobin is to any degree oxygenated the larvae irrigate most of their time, and throughout their irrigation the haemoglobin must be giving up oxygen to the tissues. Its functional significance under these conditions can remain in no doubt since carbon monoxide-treated larvae under similar circumstances behave as if in complete oxygen lack. At progressively higher oxygen concentrations the haemoglobin gradually becomes fully oxygenated during irrigation, but the increased length of time which this takes in an active animal as compared with an immobile one shows that during the irrigation activity the haemoglobin is acting in oxygen transport. This must be on account of the muscular activity of irrigation. The haemoglobin's transporting ability will make possible not only a more aerobic muscular activity but also a more rapid recovery from any preceding oxygen lack.

The irrigation behaviour of larvae after a prolonged period of anaerobiosis is essentially similar to that after a short pause in that it is concerned with recovery from oxygen lack, but owing to the greater length of anaerobiosis involved, it is more violent and prolonged in nature. During such recovery the possession of haemoglobin considerably lessens the recovery time. In this case the haemoglobin seems to be functional even in fully aerated water, for with carboxyhaemoglobin the recovery of larvae is considerably slower.* At lower oxygen concentrations the retarding effect of lack of haemoglobin is even more marked, and below 15% air saturation recovery is no longer possible. Without haemoglobin one would suppose that the larvae could not live for any prolonged length of time at oxygen concentrations below this value, since inability to maintain or return to aerobic metabolism would prevent any feeding or in fact any activity. Harnisch (1936), from studying the metabolism of larvae recovering from oxygen lack, also arrived at the conclusion that the main significance of the haemoglobin lay in hastening recovery by acting as an oxygen transporter.

The significance of haemoglobin in keeping the animals aerobic, and therefore active at low oxygen concentrations, was also evident from the behaviour of my carbon monoxide-treated larvae at constant low oxygen pressures: at all oxygen concentrations lower than almost fully air-saturated water the proportion of motionless individuals was higher than normal, while at 10% air saturation the animals behaved as if anaerobic.

Finally, a further noticeable effect of carbon monoxide was in reducing the amount of filter-feeding. Normal larvae can only use this method when oxygen is present and the inability of larvae with carboxyhaemoglobin to filter-feed at low oxygen concentrations must be due to their low internal oxygen pressure without the functional blood pigment. A second possible interpretation of this effect of carbon monoxide is that the gas might have a direct inhibitory effect on some step in the secretory activity of the salivary gland cells, as it is known to have on cytochrome oxidase, and it would thus be by eliminating the net-spinning part of the cycle that filter-feeding is made impossible. Arguments against this are that the carbon monoxide pressure of the water during experiments was very low, and since it was kept at one-sixth of the oxygen present in the water, was lowest in the conditions under which inhibition of feeding was most marked, and secondly, that during the rather common atypical filter-feeding of carbon monoxide-treated larvae nets were spun in the usual manner but the larvae failed to perform the irrigation part of the cycle. It would seem then that oxygen is needed rather for the rapid undulatory activity during feeding than for the salivary secretion.

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* The extension of the range of function of the pigment to oxygen pressures higher than those found by Ewer can be explained by the increased metabolism of the larvae due to high muscular activity and post-anaerobic recovery processes.

In its general mode of life *Chironomus* shares certain features with other tubicolous, haemoglobin-bearing invertebrates. Most like it in habits is the marine littoral echiuroid, *Urechis caupo*, for not only does this animal irrigate its burrow for respiratory purposes but also filter-feeds by means of a mucous cone attached to the head and filled by its irrigation activity (Fisher & MacGinitie, 1928). Like *Chironomus*, too, its intermittent irrigation tends to be irregular, with pauses varying from about 20 min. to more than an hour in length (Hall, 1931). In *Urechis*, however, the relatively greater total quantity of haemoglobin provides an oxygen store lasting approximately 70 min., so that such rests do not entail anaerobiosis. At low tide there is evidence that the haemoglobin not only functions as an oxygen store but also acts as an oxygen transporter during the greater part of the time that the burrows are exposed (Redfield & Florkin, 1931). Thus the possession of haemoglobin, by both storing oxygen and transporting it at low pressures, enables *Urechis* to lead an aerobic life when aerated sea water is not available. Much the same may be said about the haemoglobin of *Arenicola*; the pauses between the animals' irrigation activity may last up to an hour (Van Dam, 1938), and it experiences low oxygen pressures during low tide. Hence in the past the storage value of its haemoglobin has been most emphasized (Barcroft & Barcroft, 1924; Lindroth, 1943). Borden (1931), however, considers it also of significance in oxygen transport at low pressures, and the animal's habit of trapping air bubbles over the gills when unable to circulate its tube water (Wells, 1945) might also indicate a transport function.

The behaviour of *Nereis virens* more closely resembles that of *Chironomus* in that the pauses between respiratory irrigation periods are longer than the oxygen storage time of the haemoglobin (Lindroth, 1938). Thus the animal normally experiences some anaerobiosis. Lindroth concludes that irrigation is controlled by the production of some metabolic substance, and that it not only serves to renew the oxygen supply but also makes possible repayment of the oxygen debt incurred during the previous pause, as I believe to be the case in *Chironomus*. The significance of the haemoglobin he thinks to lie in the pigment storing a small amount of oxygen and thereby reducing the duration of anaerobiosis during pauses. His reasons for rejecting a transporting function, however, suffer from being based largely on theoretical considerations unsupported by experimental evidence such as estimation of the metabolism of animals with carboxyhaemoglobin would afford.

In tubicolous animals, where external and internal oxygen pressures fluctuate normally from air saturation to very low values, it would seem to be impossible for haemoglobin to function exclusively as either transporter or store of oxygen unless the animal has the power to cut itself off suddenly and completely from its external oxygen supply, in which case its transporting ability would be eliminated. The reduction of circulation in the retia respiratoria in *Nereis* during a pause (Lindroth, 1938) may accomplish this (although the rhythm does not slow much during the first 10 min. of the still period and could well therefore be transporting during that time), but the trapping of air bubbles by the animal at low tide indicates some continued connexion with external oxygen during this time, and the possibility of the haemoglobin functioning in oxygen transport. And *Nereis* is the only haemoglobin-containing animal in which such a reduction of circulation during immobility has been reported, although the chlorocruorin-containing *Sabella* is known to stop its circulation when the crown is withdrawn into the tube (Fox, 1933). In *Chironomus* the heart continues to beat at a slower rate than during activity, but sufficiently to maintain a circulation. Thus in general, unless there is experimental evidence to the contrary, invertebrate haemoglobins must be considered as oxygen transporters as well as stores.

The experiments described above indicate that for *Chironomus* at any rate the transporting ability of haemoglobin has greater significance in the animal's life than its storage value, for as an oxygen transporter the haemoglobin can, and does, function for as long as the animal is irrigating at low oxygen pressures, and thus makes possible activity under these circumstances, while at higher oxygen pressures it provides a quick oxygen supply for post-anaerobic recovery processes and for the high muscular activity of respiratory irrigation and filter-feeding. As a store it reduces the amount of anaerobiosis to be undergone during short periods of inactivity and so lengthens the time during which the larva can remain still within its tube for a given accumulation of anaerobic metabolic by-products.

Thus by increasing the proportion of aerobic to anaerobic metabolism, as an oxygen transporter it allows the animal to be economically active and as an oxygen store it allows it to be economically lazy. Were the animal a little more active in its irrigation behaviour, in other words if it irrigated at more frequent intervals, it could, thanks to its haemoglobin, lead a more aerobic existence than it does. The capacity of *Chironomus* to live anaerobically is higher than that of most aquatic organisms, indeed *C. plumosus* is one of the few animals which can live at the bottom of certain eutrophic lakes where extensive summer and winter oxygen depletion occurs (Thienemann, 1923). Harnisch (1938, 1939) has shown that during sustained periods of anaerobiosis the fatty acids produced are converted into fats and stored in the fat-body; this persists in the non-feeding adult (Possompès, 1937) and forms, as in many insects, one of the chief forms of stored energy. This anaerobic metabolism, although wasteful of glycogen, serves the purpose of forming fat, and this may explain the persistence of some degree of anaerobiosis in larvae even when oxygen is available to them in the water above their tubes. As regards the greater quantity of glycogen necessary for anaerobic energy, it should be pointed out that *Chironomus*, living in organic mud with a rain of plankton from above, need never be short of food material. Thus periods of obligatory or self-imposed anaerobiosis can be alternated with aerobic metabolism accompanied by feeding activity; it is significant that after the long periods of oxygen lack to which my larvae were subjected, all the normal animals adopted filter-feeding once oxygen was again available to them. In this the haemoglobin would appear to be of paramount significance in allowing for quick recovery and optimal feeding, even at low oxygen concentrations.

SUMMARY

1. The behaviour of final-instar larvae of *Chironomus plumosus* housed in U-shaped glass tubes was observed at various concentrations of dissolved oxygen and carbon dioxide.

2. Respiratory behaviour, consisting of intermittent irrigation of the tube, alternates with periods of filter-feeding or complete immobility. In well-aerated water about 50% of the time is occupied by respiratory behaviour, 35% by filter-feeding and the remainder by periods of rest. As the oxygen concentration in the water drops, progressively less time is occupied by filter-feeding and immobility and more by respiratory irrigation. Below 10% air saturation of the water larvae no longer feed. When placed in completely anaerobic conditions larvae at first irrigate intermittently but subsequently relapse into immobility.

3. During respiratory behaviour the amount of irrigation and the length of pauses between periods of irrigation change at different oxygen and carbon dioxide contents of the water in such a way as to suggest that the respiratory irrigation is controlled by internal pH changes in the larvae.

4. A spectroscopic examination of the haemoglobin in living larvae showed that the blood pigment holds an approximately 9-min. store of oxygen for the resting animal. In addition to this it acts in the transport of oxygen from the tube water to the larval tissues when the larva pauses between periods of irrigation. It thus decreases the amount of anaerobiosis to be endured during short periods of inactivity. Nevertheless, larvae without a functional haemoglobin (i.e. with carboxyhaemoglobin) still continue to pause during their respiratory behaviour, and the pauses are not strikingly curtailed in length.

5. At very low oxygen concentrations (7·5–9·0% air saturation), when the larva irrigates the tube almost unceasingly, the haemoglobin remains in a state of partial oxygenation, during which time it is functioning continuously in oxygen transport. At these oxygen concentrations larvae with carboxyhaemoglobin do not show respiratory activity but assume the immobility characteristic of anaerobic conditions.

6. Larvae with carboxyhaemoglobin tend to be less active than normal animals, except in well-aerated water, the decreased activity being largely due to a reduction in the amount of filter-feeding. Such larvae have not been observed to filter-feed at oxygen concentrations below 26% air saturation, whereas the limiting concentration for normal larvae is 10%.

7. After a prolonged period of anaerobiosis larvae show evidence of the repayment of an oxygen debt by prolonged irrigation of the tube when oxygen is once more available. A return to a normal irrigation rate is rapid and is usually followed by a period of filter-feeding The rate of recovery is proportional to the oxygen content of the incoming water, but normal larvae can recover even in water only 7% air saturated. Larvae with carboxyhaemoglobin, on the other hand, show a considerably

retarded rate of recovery from anaerobic conditions, and cannot recover in water less than 15% air saturated.

8. The main significance of haemoglobin in the life of a full-grown *Chironomus* larva would thus seem to be threefold: (*a*) haemoglobin enables the larva to maintain the active process of filter-feeding when relatively little oxygen is present; (*b*) it acts in oxygen transport at very low oxygen concentrations, thereby enabling continued respiratory irrigation; and (*c*) it greatly increases the rate of recovery from periods of oxygen lack, making such recovery possible even under adverse respiratory conditions.

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THE RETENTION OF SALT AND THE REGULATION OF THE NON-PROTEIN NITROGEN FRACTION IN THE BLOOD OF THE AQUATIC LARVA, *SIALIS LUTARIA*

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(With Five Text-figures)

INTRODUCTION

There seems little doubt that the low permeability of the cuticle of fresh-water insect larvae is an important factor in preventing loss of salt to the environment. In several species, however, it has been shown that the salt leakage is comparatively rapid, and that this is continuously balanced by an active uptake of ions from the surrounding fresh water. This is also characteristic of many fresh-water animals (Krogh, 1939). Krogh originally demonstrated this mechanism by first reducing the blood salt concentration by washing the animals in continuously running glass-distilled water. These animals, when replaced in tap water or very dilute salt solutions, actively absorbed ions from these solutions.

We have recently applied this technique to a variety of Crustacea and insect larvae, and have found that certain species, of which *Sialis lutaria* is one, can withstand washing in distilled water for many weeks and still maintain an adequate blood salt concentration. In view of the large surface area of the paired tracheal gills of *Sialis* larvae this result is at first sight rather surprising, for in the mosquito and *Chironomus* larvae there is good evidence that the anal gills are regions of high permeability, as well as the seat of the active salt-absorbing mechanism (Koch, 1938; Wigglesworth, 1938). We therefore set out to discover whether *Sialis* possesses any such salt-absorbing mechanism and to investigate further the mechanism of salt retention.

We have also investigated the possibility of a mechanism regulating the concentration of some of the organic substances in the blood which might compensate for any changes in its salt content. Such a mechanism could obviously provide a means of osmotic regulation of great importance to fresh-water animals. The possibility of the existence of such a mechanism was suggested by the work of Wigglesworth (1938) on the larva of *Aëdes aegypti*. The larvae of this species, when reared in distilled water, had a blood chloride concentration somewhat lower than normal. After the animals were transferred to tap water the blood chloride rose rapidly during the first day, but there was no corresponding rise in the total osmotic pressure. This suggested that there had been a compensatory reduction in the non-chloride fraction of the total osmotic pressure. There was also some evidence of a reduction in the non-chloride fraction when the blood chloride was increased by immersing the animals in hypertonic salt solutions.

It is characteristic of insect blood that the chloride concentration is low and that a high proportion of the non-chloride fraction is made up of amino-acids. To this, as will be seen, *Sialis* is no exception, and therefore a compensatory regulation of the non-chloride fraction would presumably involve a regulation of these amino-acids. *Sialis* was chosen as an easily obtainable aquatic larva, from a single specimen of which it was possible to extract enough blood for the necessary estimations.

METHODS

The larvae were caught in small lakes and ponds in south Northumberland by sifting the mud dredged from within a few feet of the edge. From about July to January proved to be the best time for catching the large penultimate stage larvae, and these were used as far as possible for the blood analysis. Although there was considerable variation between the bloods of different individuals we could find no significant difference between the bloods of different instars.

Blood was extracted from the larvae after they had been narcotized in water through which CO_2 was being bubbled. On removal from the water the larvae remained immobilized long enough for their blood to be extracted easily. The larvae were dried with filter-paper and a little acetone was applied with a paint brush to the surface of the pronotum. After the acetone had evaporated a coat of bees-wax dissolved in ether was applied. This gave a hydrofuge surface over which blood would not spread. The centre of the pronotum was then pricked with a needle and the blood, as it emerged in the form of a discrete drop, was collected in a fine glass capillary pipette waxed at the tip and operated with a rubber tube and suction mouthpiece. The blood was squeezed out, pressure being applied by rolling a glass rod over the larva from behind forwards. The larvae can survive very rough treatment and would recover after all possible blood had been squeezed from them by this 'rolling pin' technique. In the experiments on blood regeneration, which are described below, as much as 25% of the body weight or approximately half of the total blood was commonly extracted without permanent harm. The extracted blood was centrifuged in small tubes of about 2 mm. bore to remove solid material.

The larvae were weighed to the nearest 0·5 mg. on a 500 mg. torsion balance. The majority weighed from 70 to 110 mg.

Total osmotic pressure of the blood was determined by the Baldes thermoelectric method which with our installation was sensitive to the nearest 0·02% NaCl equivalent.

Protein and non-protein nitrogen were determined by a Kjeldahl method already described (Shaw & Beadle, 1949). For the quantities estimated (1·0–10·0 μg.) the standard deviation was 0·02–0·04 μg. N. The osmotic equivalent of the non-protein nitrogen-containing compounds was calculated on the assumption that each molecule contained 1 atom of nitrogen. This would give a maximum figure and might therefore be too high.

There was usually more than enough blood available from each larva to enable us to make duplicate estimations by each of the above three methods, each of which requires less than 1 μl. of blood.

The total chloride in the body was estimated in the following way. Weighed larvae were each placed in a crucible containing about 3 ml. conc. HNO_3, together with 30 μl. of 2% $AgNO_3$ delivered quantitatively from an internally waxed micropipette of the Wigglesworth type. The crucibles were covered and digestion was completed overnight at room temperature. After the addition of about 5 ml. of acetone and three drops of saturated iron alum solution as indicator, the excess $AgNO_3$ was estimated by titration with approximately equivalent strength NaSCN from a Heatley-Rehberg type microburette of 50 μl. capacity, stirring with a fine glass rod. It was essential to control each set of estimations by running simultaneous samples of (*a*) 30 μl. of 2% $AgNO_3$ alone, and (*b*) 30 μl. 2% $AgNO_3$ plus 30 μl. standard 0·5% NaCl, by which the strength of the NaSCN solution was checked on each occasion. The total chloride in normal larvae ranged from 0·060 to 0·090 mg. NaCl/100 mg. body weight. With the above method of control the results were accurate to the nearest 0·002 mg. NaCl/100 mg.

The method and apparatus used for exposing the larvae and other animals to continuously flowing glass-distilled water will be described in another paper. The distilled water used in these experiments was shown to contain less than 0·0002 mM-NaCl. This was the lowest concentration which we could detect as a faint cloudiness in a glass tube 1 cm. in diameter and about 50 cm. long after the addition, together with the distilled water, of about 3 ml. of $AgNO_3$ solution acidified with nitric acid. Comparison was made with a similar tube of distilled water with no added chloride. The Newcastle tap water in which the larvae were kept contained approximately 0·3 mM-NaCl.

PERMEABILITY TO CHLORIDE AND RETENTION DURING STARVATION

The normal concentration of chloride in the blood ranged from 0·15 to 0·34% NaCl, and the total osmotic pressure from 0·80 to 1·10% NaCl. It was demonstrated that the cuticle is permeable to chloride by immersing specimens in a solution of 1% NaCl. They remained healthy in this solution almost indefinitely, but showed a considerable increase of blood chloride during the first few days. We did not analyse this in detail, but the results of experiments done at different times are given in Table 1A. Temperatures were not recorded but the environment was an unheated room. The more rapid penetration in July 1947 was no doubt connected with the high temperatures at that time.

To ensure that the chloride was not taken in through the gut under these conditions, larvae were ligatured with fine silk thread round the neck and at the base of the tail process in front of the anus. After 3 days (in September 1948) in 1% NaCl the chloride was clearly higher than in ligatured controls in tap water (Table 1B).

It was shown that the tracheal gills form a high proportion of the permeable surface of the larva. The gills were removed with fine scissors whilst the larva was under CO_2 narcosis and without gills the animal will survive indefinitely provided that the water is freely circulating to ensure an adequate supply of oxygen. A plug forms rapidly over each wound, which soon blackens. After 3 weeks of starvation in running tap water, the blood chloride had dropped somewhat (Table 1 C). Two were then treated for 24 hr. with 1 % NaCl together with two unoperated controls. The figures, though few, show that the rapid penetration of chloride into normal larvae is much retarded, though not eliminated by removal of the gills.

Table 1

Previous treatment	Date	Medium	Duration of experiment (days)	Blood chloride (% NaCl)
A. None	July 1947	1 % NaCl	2	0·59, 0·79, 0·75
	—	—	5	0·82, 0·89, 0·83, 0·66
	Nov. 1947	—	1	0·32, 0·36
	—	—	7	0·43, 0·49
	July 1948	—	16	0·65, 0·70, 0·75
B. Ligatured	Sept. 1948	1 % NaCl	3	0·35, 0·40
	—	Tap water	3	0·17, 0·19 (Control)
C. Gills removed	Jan. 1948	Tap water	21	0·11, 0·17, 0·07, 0·15, 0·12, 0·13
	—	1 % NaCl	1*	0·15, 0·24
None	—	—	1*	0·37, 0·56 (Control)

* After 21 days in tap water.

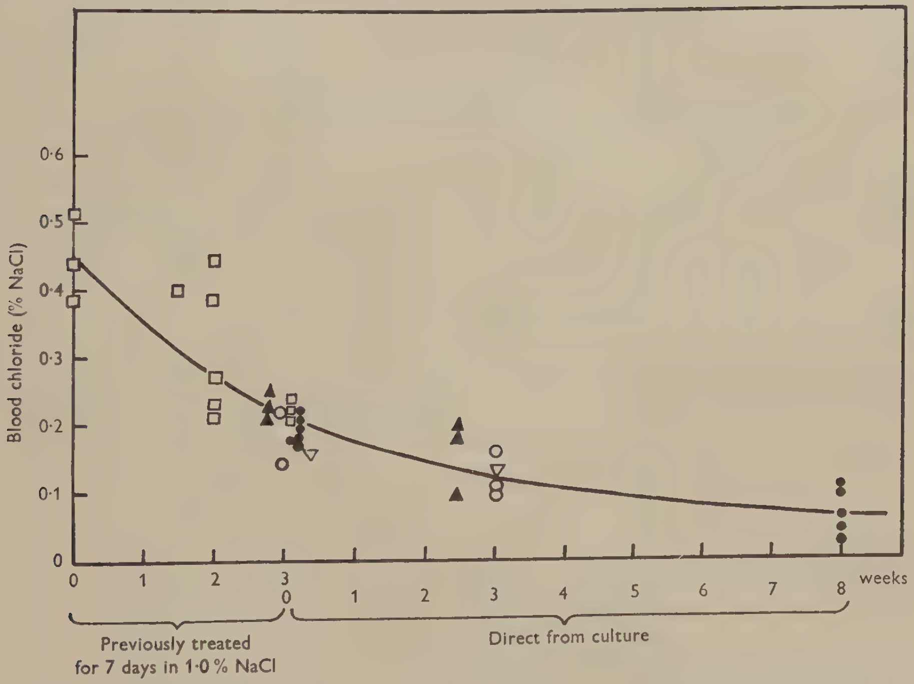

Fig. 1. Loss of blood chloride during prolonged starvation in flowing glass-distilled water or tap water. ☐ Into tap water after 7 days in 1 % NaCl. ● Direct to tap water. ○ Direct to distilled water. ▲ Direct to distilled water. ▽ Direct to distilled water.

The general body surface, and especially that of the tracheal gills, is therefore permeable to chloride diffusing inwards. Outward diffusion was studied by prolonged starvation in running tap water and glass-distilled water. The results of five separate sets of experiments are reproduced in Fig. 1, where they are combined to give a single curve. In one set the blood chloride had previously been boosted by treatment with 1 NaCl for 7 days. Thereafter, the larvae were washed in running tap water, and within 3 weeks the blood chloride had returned to the normal level. The rest of the curve was drawn through points derived from experiments on untreated larvae, one set being washed for 8 weeks in tap water, the remainder in glass-distilled water for periods up to 3 weeks. It is clear that the cuticle is permeable to chloride diffusing outwards and that the greater the difference between internal and external concentrations the more rapid is the diffusion. The fact that retention is as effective in distilled as in tap water suggests that active uptake of ions from the normal environment does not take place in this species. Further evidence for this conclusion will be presented later (p. 102). The shape of the curve suggests that the rate of loss of chloride is approximately proportional to the difference in concentration between external and internal medium. It therefore seems likely that the retention of salt during prolonged starvation is merely the consequence of the extremely low permeability of the integument.

THE CHLORIDE BUDGET DURING REPEATED BLOOD EXTRACTIONS

Since the blood chloride is only very slowly reduced by prolonged washing with distilled water, we could get no certain evidence by this method concerning the presence or absence of an active ion-absorbing mechanism. We found, however, that after a large proportion of the blood had been removed and the animal returned to water, the blood volume was regenerated in a short time. This treatment could be repeated several times on the same larva. By following the changes of weight and of blood chloride concentration during this treatment and by estimating the total chloride left in the larva at the end we were able to calculate whether chloride had been absorbed from the medium. We were incidentally able to arrive at a figure for the blood weight as a percentage of the total weight of the larva.

Fig. 2 shows the weight and blood chloride concentration changes of two larvae in tap water and in distilled water, both continuously flowing. Blood from these larvae was extracted three times during 2 weeks. These results are typical of several which showed essentially the same features. After each extraction the weight was partially restored by absorption of water. About 40% of the original body weight was extracted during the first two operations and water amounting in all to about 25% was absorbed during the recovery periods. If an active ion-absorbing mechanism were present it should come into operation in these conditions when an inflow of water, following a blood extraction, brings about a reduction in the blood chloride concentration. The fact that the drop in blood chloride concentration was as great in tap water as in distilled water (Fig. 2) suggests that no such absorption was taking place, but a quantitative estimate was required to establish this point.

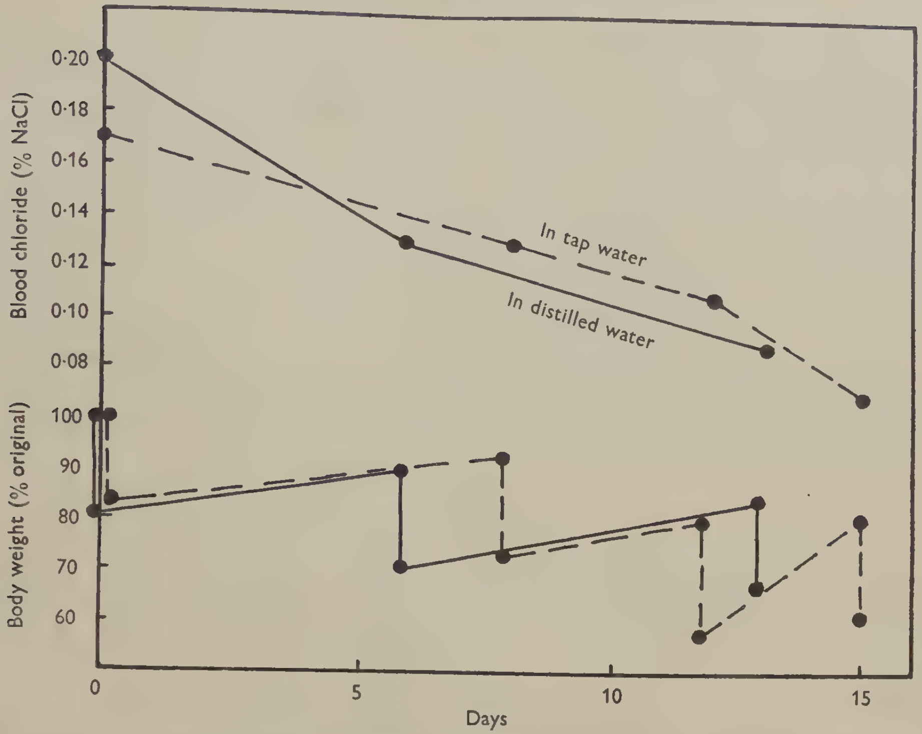

Fig. 2. Effect of repeated blood extractions, at intervals of several days, on the weight and blood chloride of two larvae.

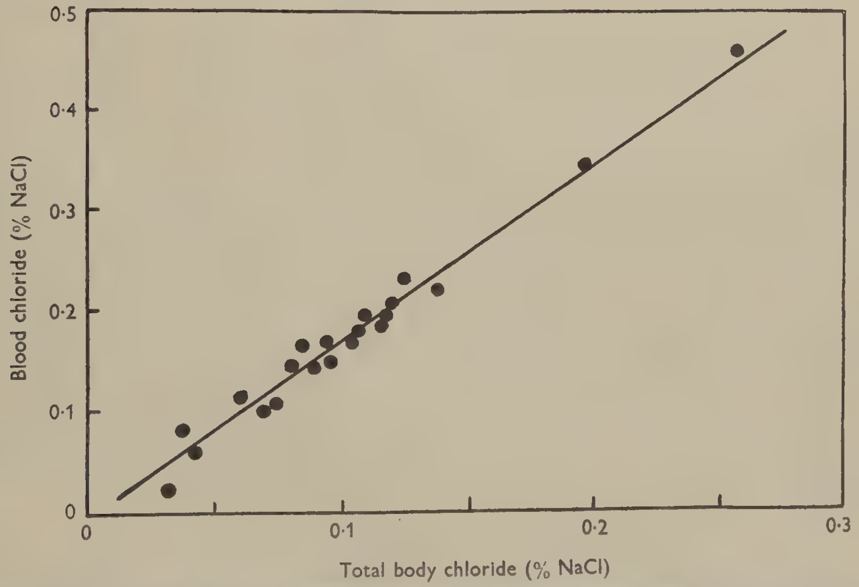

Fig. 3. Relation between the concentration of chloride in the blood and that in the whole body. Low blood chlorides induced by prolonged starvation in tap water, high blood chlorides by treatment with NaCl solutions up to 1 %.

For this it was necessary to know the total chloride in the body of the larva before the start of the experiment. Owing to a very great individual variation an accurate estimate of this value could not be made from the mean of a number of other specimens, but fortunately it was discovered that the blood chloride concentration was directly proportional to the total body chloride concentration over a wide range of blood chloride concentrations (Fig. 3). This relation could be explained by assuming (*a*) that the chloride is mainly confined to the blood and is not found in the tissues in any significant quantity, as is the case in vertebrate muscle, and (*b*) that the blood weight as a percentage of the body weight is approximately constant at about 56 %, as given by the slope of the line in Fig. 3, that is, about 56 % of the body weight. Therefore a knowledge of the blood chloride concentration estimated in a small sample of extracted blood enabled us to read off from this curve the total body chloride and thus obviate a direct analysis of the whole larva at the start.

Table 2

Medium	Duration of experiment (days)	No. of extractions	Original blood chloride (% NaCl)	Total extracted chloride (NaCl % original body wt.)	Final total body chloride (% NaCl)	Extracted + final chloride	Calculated original chloride*
Distilled water	8	3	0·14	0·078	0·018	0·096	0·088
	15	4	0·17	0·090	0·019	0·109	0·110
	15	4	0·12	0·050	0·023	0·073	0·077
Tap water	7	3	0·22	0·075	0·046	0·121	0·130
	7	4	0·16	0·090	0·008	0·098	0·099
	13	3	0·19	0·079	0·028	0·107	0·115
	13	3	0·19	0·084	0·034	0·118	0·115
	13	3	0·22	0·099	0·039	0·138	0·130
	13	3	0·22	0·092	0·028	0·120	0·130
	13	3	0·20	0·084	0·036	0·120	0·120
	13	3	0·19	0·089	0·030	0·119	0·115

* From Fig. 3.

Table 2 summarizes some experiments involving repeated extractions in which the complete chloride budget was computed in each case. The total chloride in the extracted blood was calculated and added to the total body chloride of the larva at the end of the experiment, and this was compared with the original total chloride estimated from Fig. 3. Three experiments were done with flowing distilled water as the external medium from which there could be no question of active uptake, and these experiments involved a maximum of four extractions over a period of 15 days. The figures in the last two columns should be identical provided there was no leakage of salt from the wound and that the normal outward diffusion was negligible during this period (Fig. 1). In fact, they approximated rather closely, and we may conclude that no significant leakage occurred. Eight similar experiments were done with tap water (Table 2). The close correspondence between the figures in the last two columns, in spite of the opportunities for error involved in the method, can be accepted as evidence against a mechanism for active uptake of chloride. If such a mechanism had been in operation the sum of the extracted chloride plus the final body chloride should have exceeded the estimated original. The same technique applied to the fresh-water crustacean, *Gammarus pulex*, which we have shown by the 'washing out' method, can actively absorb chloride, showed a definite excess of extracted chloride plus final chloride over the original chloride. These and related experiments on other animals will be described in another paper.

The same result was obtained in another way in later experiments on repeated extractions of blood with the larvae living in tap water (Table 4A). From the weight of blood extracted and from the increase in weight of the larva as the blood was regenerated, the new blood chloride concentration could be calculated assuming (*a*) an initial blood weight of 56% of the body weight, and (*b*) the absence of any significant leakage or active absorption of chloride during the period (13 days); and assuming that the increase in weight was due to the uptake of pure water. The identity (to the first two decimal places) of the calculated with the observed blood chloride concentration again supports the conclusion that no active absorption of ions takes place under these conditions.

The same calculation was applied to similar experiments in 0·5% NaCl (Table 4B), assuming that the weight increase was due to the uptake of 0·5% NaCl. This assumption is not of course valid in view of the low permeability to salt, but the result is interesting. After the first operation when the concentration gradient between the external and internal medium was still high, and salt must have been penetrating relatively rapidly, the calculated and observed blood chloride concentrations were about equal. Thereafter the gradient was less steep, the fluid taken up was presumably more dilute than 0·5% NaCl, and the observed was significantly lower than the calculated blood chloride.

OSMOTIC REGULATION OF THE BLOOD

There is no evidence from the preceding sections of any active control of the salt content of the blood. The rate of loss by diffusion is slow due to the extremely low permeability of the integument. On the other hand, there are signs of a mechanism which controls the total osmotic pressure of the blood and compensates for fluctuations in the salt content by regulating the amount of some other constituent. When the blood chloride concentration is artificially altered, the total osmotic pressure is not correspondingly affected, which implies that there is some compensatory regulation of what might be called the 'non-chloride' fraction of the total osmotic pressure.

It has already been shown that the reduction of the blood chloride concentration by starvation is a lengthy operation, and, since the concentration is low at the start, no drastic change can be induced. The reduction in the concentration of chloride in the blood due to 6 weeks' starvation in tap water resulted in, either no fall in the total osmotic pressure or a fall in the total osmotic pressure, but none in the osmotic pressure of the non-chloride fraction (Table 3). The regulation of the non-chloride fraction was more clearly demonstrated when the blood chloride level was increased to about three times the original value by immersing the larvae for about 7 days in 1% NaCl (Table 3). The mean rise in osmotic pressure of the blood was relatively small, from which it follows that there was a considerable fall in the non-chloride fraction.

Table 3. *Percentage* NaCl *equivalents*

	Chloride	Osmotic pressure	Non-chloride fraction (difference)
A. Controls	0·21	1·11	0·90
	0·24	1·01	0·77
	0·34	1·09	0·75
	0·34	0·99	0·65
	0·27	1·04	0·77
	0·19	0·84	0·65
	0·19	1·00	0·81
	0·22	0·98	0·76
Mean	0·25	1·01	0·76
B. 6 weeks' starvation in tap water	0·10	1·01	0·91
	0·04	0·79	0·75
	0·10	0·80	0·70
	0·06	1·01	0·95
	0·02	0·69	0·67
Mean	0·06	0·86	0·80
C. 7 days in 1 % NaCl	0·59	1·12	0·53
	0·79	1·15	0·36
	0·73	1·02	0·29
	0·82	1·09	0·27
	0·56	0·94	0·38
	0·65	1·18	0·53
	0·70	1·27	0·57
	0·76	1·31	0·55
Mean	0·70	1·13	0·43

REGULATION OF NON-PROTEIN NITROGEN

By means of an ultramicro-Kjeldahl method already described (Shaw & Beadle, 1949), which includes the separation of protein from non-protein nitrogen, it was found that the osmotic pressure exerted by the non-chloride fraction of the blood of *Sialis*, like that of many other insects, was due largely to non-protein nitrogen compounds dissolved in the blood. We therefore set out to find evidence for the compensatory regulation of these substances.

In the first place, we found during prolonged starvation of the larvae in tap water, which as noted above, caused some reduction in the blood chloride level, the concentration of the non-protein nitrogen, like that of the non-chloride fraction, was maintained at the normal level (Fig. 4). During this starvation period, however, there was a very striking drop in the blood protein level presumably due to the metabolism of the protein in the absence of food. The non-protein nitrogen must, therefore, be either not metabolized or maintained at the expense of some other nitrogen source.

Evidence for a compensatory regulation of the non-protein nitrogen fraction was obtained from experiments on the regeneration of the blood following repeated extractions. The mean figures for six experiments in tap water are given in Table 4A. The blood chloride concentration after two extractions was less than half the original, but the drop in osmotic pressure was comparatively slight. The non-protein nitrogen level was seen to have actually increased during these experiments in spite of the extraction of a considerable amount in the two operations. From the weight of blood extracted after each operation, and the subsequent increase in weight of the larva when replaced in water, it was possible to calculate the resulting concentration of non-protein nitrogen, assuming a blood weight of 56% of the body weight (p. 102), if no regulation had taken place. This was found to be 161 mg.%, and thus less than half the concentration actually found, and therefore there is little doubt that a large quantity had appeared in the blood. A similar calculation also showed that the observed fall in protein nitrogen would approximately account for the extra non-protein nitrogen which had appeared at each stage (Table 4A, last two lines).

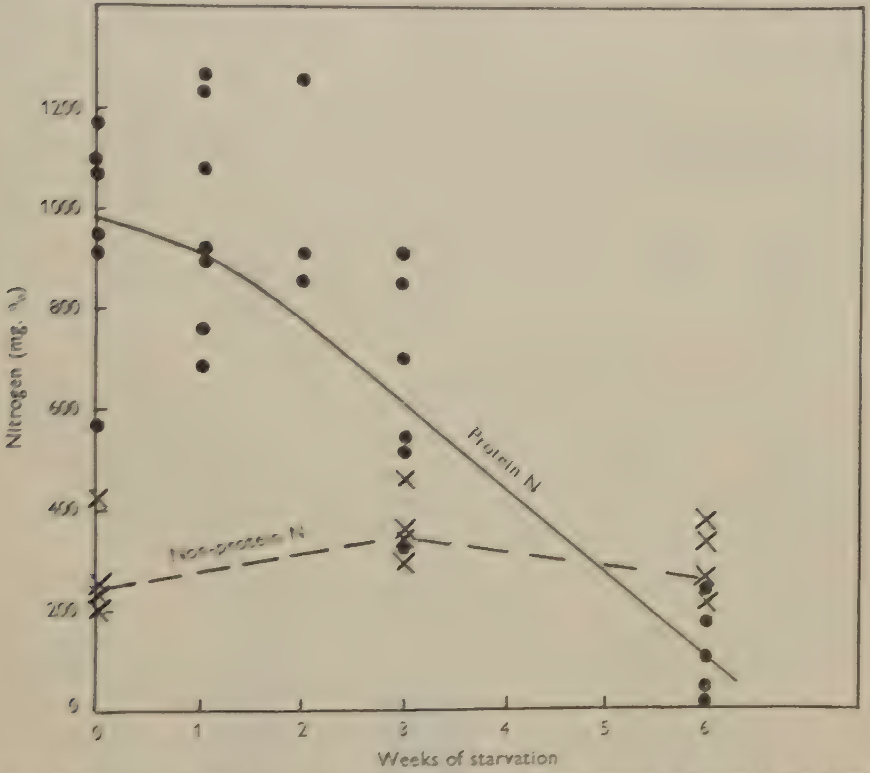

Fig. 4. Effect of prolonged starvation on the protein and non-protein nitrogen in the blood.

In further experiments after the blood extractions the larvae were put into 0·5% NaCl. The blood volume was regenerated as before but there was a rise in the blood chloride concentration without any significant change in the total osmotic pressure of the blood (Table 4B). As before, the non-protein nitrogen level after each regeneration was greater than the calculated value, assuming no regulation to have taken place. Thus, in this case also non-protein nitrogen had been added to the blood. However, in each case the new non-protein nitrogen level was significantly lower than the original, indicating that the concentration of chloride in the blood

Table 4

	A. Tapwater (mean of 6)			B. 0·5 % NaCl (mean of 6)		
	Start	6 days	13 days	Start	6 days	13 days
Initial wt. (mg.)	86	—	—	81	—	—
Calc. initial blood wt. (mg.)	48	—	—	45	—	—
Blood extracted (mg.)	16	19	—	19	23	—
Water uptake (mg.)	—	10	11	—	21	22
Blood Cl (% NaCl)	0·20	0·15	0·09	0·14	0·31	0·36
Calc.* blood Cl (% NaCl) (see p. 103)	—	0·15	0·10	—	0·30	0·40
Blood osmotic pressure (% NaCl)	0·91	0·86	0·72	0·97	0·99	0·96
Non-protein N (mg. %):						
Found	243	268	364	273	200	151
Calc.*	—	186	181	—	152	104
Protein N (mg. %):						
Found	980	657	241	802	222	87
Calc.†	—	663	262	—	396	69

* As diluted by extraction and subsequent uptake of water (A) or of 0·5% NaCl (B) only.

† Diluted as above and by breakdown of amount equivalent to the excess of found over calculated non-protein nitrogen.

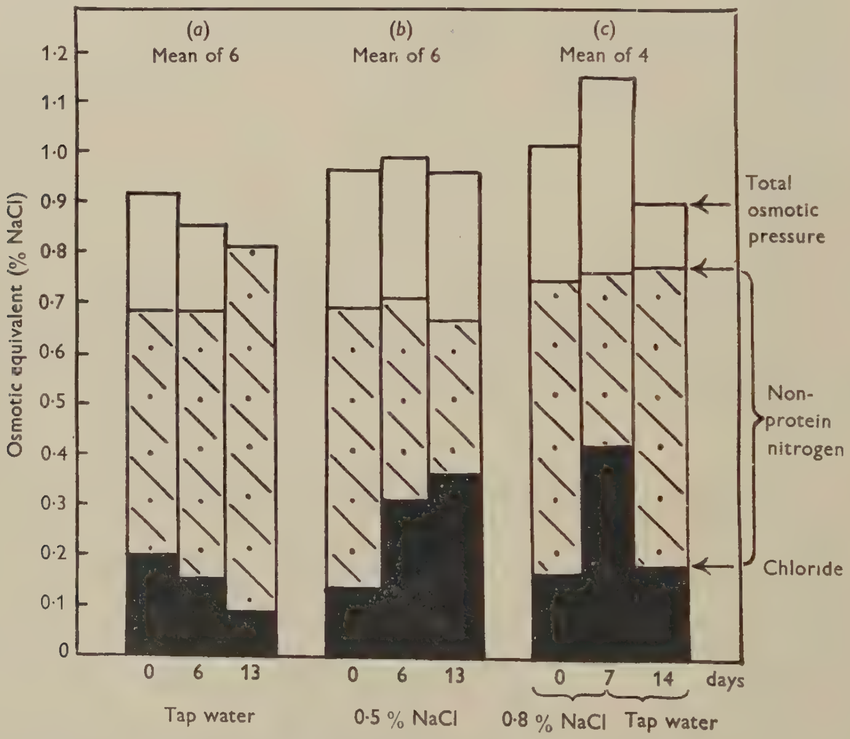

Fig. 5. Summary of experiments on the relation between blood chloride and non-protein nitrogen: (*a*) increase in non-protein nitrogen with decrease in chloride; (*b*) decrease in non-protein nitrogen with increase in chloride; (*c*) alternate decrease and increase in non-protein with increase and decrease of blood chloride respectively. Each set of experiments was performed on one set of larvae.

controlled the amount of non-protein nitrogen that was added. In these experiments the protein nitrogen was reduced after the first operation much more than could be accounted for by dilution and breakdown to provide the added non-protein nitrogen.

The results of these experiments are more clearly summarized by the histograms in Fig. 5*a*, *b*, in which the osmotic equivalent of the non-protein nitrogen has been calculated as % NaCl on the assumption that the molecules in question (presumably amino-acids) each contained 1 atom of nitrogen. The compensatory regulation of the non-protein nitrogen fraction is very clearly demonstrated, and was further confirmed by a final set of experiments in which the chloride was alternately raised and lowered in the same batch of four larvae by blood regeneration: first, in 0·8% NaCl and then in tap water. The inverse changes in the non-protein nitrogen fraction are very obvious (Fig. 5*c*).

DISCUSSION

There is little doubt that the larva of *Sialis lutaria*, unlike some insect larvae and many other fresh-water animals, is unable to absorb chloride actively from the medium in which it is living. This is presumably true for other ions as well. The cuticle, including that of the tracheal gills, is sufficiently impermeable to enable the larva to withstand prolonged starvation without undue loss of salt. Radioactive tracers might well be used to obtain further evidence on this point.

It is perhaps significant that *Sialis* has no anal gills, which in some insect larvae have been shown to be the site of the ion-absorbing mechanism. It would be interesting to make a comparison between the structure of the cuticle and cellular lining of such anal gills and the structure of the tracheal gills of *Sialis*.

The regulation of the non-protein nitrogen in the blood to compensate for fluctuations in the chloride concentration suggests a number of interesting problems. The source of the additional non-protein nitrogen appearing in cases of chloride depletion has yet to be established. Raper & Shaw (1948) showed that the non-protein nitrogen in the blood of the aquatic larva of *Aeshna cyanea* is contained largely in a few simple and highly soluble amino-acids. Simultaneous measurements of protein and non-protein nitrogen recorded in this paper have given figures which are not inconsistent with the view that the regulation under discussion is concerned with the equilibrium between the blood protein and the blood amino-acids. However, nothing is known of the nature of the stimulus which would bring about increased or decreased protein hydrolysis.

The normal level of the blood protein in freshly caught *Sialis* larvae was extremely variable, ranging from 1 to 12% (calculated as protein N × 6·25). The mean was 6–7%, which is about the concentration of protein found in mammalian blood. This value is high compared with estimations made so far on other insects for which values from 1·0 to 6·6% have been found (Wigglesworth, 1942, p. 229). During starvation, however, the blood protein can be reduced to less than one-twentieth of the original without any apparent effect on the health or activity of the larvae (Fig. 4). Such a reduction would be quite impossible in a vertebrate, in which the colloid osmotic pressure exerted by the plasma is an essential factor in the control of water transfer between blood and lymph. Observations and experiments on mammals, including man, show that a reduction in the blood protein by 50% causes a serious derangement of the water balance and other marked symptoms of ill health (Dicker, 1948; Beattie & Herbert, 1948).

We have made some measurements (unpublished) of colloid osmotic pressure of the blood of the fresh-water crab, *Telphusa fluviatile*, and in one instance after two successive blood extractions, the blood volume being restored by absorption of water, the colloid osmotic pressure was reduced from 33 to 2 mm. of water with no apparent effect on the activity of the crab.

Since the colloid osmotic pressure of the haemolymph is a factor to which arthropods seem to be comparatively indifferent, it would be of interest to investigate the effect of changes in this on the excretion of urine in the Crustacea. The amount of urine formed might be supposed to depend, in part, upon the excess of hydrostatic over colloid osmotic pressure of the haemolymph.

SUMMARY

1. As in the blood of other insects, chloride forms only a small proportion of the total dissolved substances in the blood of *Sialis lutaria* larvae. (Chloride 0·15–0·35% NaCl, osmotic pressure 0·80–1·10% NaCl.)

2. Only after more than 5 weeks of starvation in distilled or tap water was the blood chloride reduced to below 0·10% NaCl. With a few days' treatment in 1·0% NaCl it could be increased to 0·5% or higher.

3. The movements of chloride induced in this way were shown to occur through the external cuticle whose permeability to ions is thus very low.

4. There is no mechanism for the active uptake of ions from the external water, and the resistance to several weeks of starvation in glass-distilled water must be attributed solely to the very low permeability of the cuticle.

5. A large proportion of the non-chloride fraction of the total osmotic pressure is non-protein nitrogen, which is presumably amino-acid.

6. Large and rapid changes in blood chloride were induced by repeated blood extractions which were followed by restoration of most or all of the blood volume by the uptake of water. There was no corresponding alteration in the total osmotic pressure.

7. The concentration of non-protein nitrogen in the blood changes in such a way that the total osmotic pressure is not seriously affected by considerable dilution or concentration of the salts as indicated by chloride, which can be halved or doubled.

8. Estimations of protein nitrogen have suggested the possibility that changes in the non-protein nitrogen are made at the expense of the blood protein.

9. The mean normal protein nitrogen of the blood is approximately the same as that of mammals. But after 6 weeks of starvation it was reduced to about one-twentieth of the original, and in some cases to practically nil without apparent effect on the health of the larva. The initial level of non-protein nitrogen was, however, maintained throughout.

10. These results suggest that a compensatory regulation of the blood amino-acids is an important component of the osmo-regulatory mechanism.

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THE ROLE OF THE COELOMIC FLUID IN THE MOVEMENTS OF EARTHWORMS

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(With One Text-figure)

INTRODUCTION

The manner in which earthworms move on the surface of the soil is now well known, for a brief but substantially correct account was given as long ago as 1894 by Friedlander and again in 1901 by Bohn. Further details and an account of the method by which co-ordination of the muscular movements is brought about have been given by Gray & Lissmann (1938*a*). Darwin's (1881) account still remains the best source of information on the burrowing of earthworms, but he was chiefly concerned with their habits and only to a lesser degree with the mechanics of burrowing. He gives, however, some valuable data on the times taken by worms to burrow into different kinds of soil and confirms an observation of Perrier that the anterior end of the body of the worm is first attenuated and thrust a little way into the soil and then expanded so that the soil is pushed away on all sides. Darwin also brought forward strong evidence for the view that burrowing into very compact soils is effected by the worms literally eating their way through it.

In a previous paper (Chapman & Newell, 1947) an attempt was made to describe the functioning of the body fluid-muscular system of the lugworm, and it was shown that even in inactive worms the body-wall muscles had a tonus sufficient to maintain a distinct positive pressure in the coelomic fluids. During movement, particularly during burrowing, the internal pressures rose to much higher figures, and from these calculations could be made of the thrusts which could be exerted against the substratum. It was believed, therefore, that measurements of the internal pressures developed in earthworms during movement and a knowledge of the way in which pressure changes are distributed about the body would help in an understanding of the mechanics of movement not only of these but of other animals with a similar body plan.

An essential preliminary to an understanding of the role of the coelomic fluid in the locomotion of the earthworm is a knowledge of the morphological relations of the coelomic compartments and of the structure of the septa, particularly of their musculature and of any perforations which may exist in them. In the lugworm, where septa are absent from the greater part of the body, circulation of the coelomic fluid is in no way impeded, and during burrowing fluid is driven forwards into the more active anterior segments, thus increasing the possibility of greater forward extension and thrust when the circular muscles of the body wall contract against it. At first sight such a redistribution of the coelomic fluid would appear to be impossible in a septate animal like the earthworm, but the possibility of communication between the serially repeated coelomic compartments cannot be ruled out. Unfortunately, incomplete accounts only have been given of the septa of the lumbricidae although the septa of some oligochaetes (e.g. those of *Pheretima*) have been described in considerable detail. It was found necessary, therefore, to re-examine the septa in earthworms.

THE COELOM AND SEPTA IN EARTHWORMS

A few anatomical features of *Lumbricus terrestris* may not be out of place. As is well known the coelom of *Lumbricus* is a fairly spacious fluid-filled cavity extending throughout practically the whole length of the body. In front of segment 5, however, its volume is so reduced by a complicated musculature stretching from the pharynx to the body wall that it is virtually occluded. The coelom communicates with the exterior by three sets of apertures; the nephridiopores, the dorsal pores and the gonoducts. The nephridiopores, of which there is one pair in every segment except the first three and last one, are minute sphinctered apertures lying just anterior to the ventral chaetae; the dorsal pores, absent from the first ten segments, lie in the mid-dorsal line and open through the anterior part of each segment (not in the intersegmental position as is often stated). The oviducts have a direct opening from the general perivisceral cavity in segment 13, but the vasa deferentia lead to the exterior from the testis sacs which are coelomic compartments completely closed from the general body cavity.

Behind segment 5, and throughout the rest of the body, the coelom is divided into segmental chambers by septa running from the body wall to become inserted into the wall of the gut. Each septum consists essentially of an anterior and a posterior layer of peritoneum, between which are muscles and connective tissue. The septa also carry blood vessels, notably the dorso-subneural commissures, as well as segmental nerves. Ventrally each septum is perforated by an aperture through which passes the ventral nerve cord.

De Ribaucourt (1901) has given a relatively complete account of the gross structure of the septa in *Lumbricus* and other forms. He includes details of the histology and states that in the genus *Lumbricus* the septa are not so strongly developed as in *Allolobophora* and certain related genera. He believed that the septal muscles take origin from the longitudinal muscles of the body wall of which they seem to be extensions. Septal muscles are more pronounced near to the body wall and less so where the septa join the gut. Circular muscles are only feebly developed and lie chiefly towards the posterior face of each septum and are lacking altogether from its ventral part. Other septal muscles are not clearly described by de Ribaucourt, and the only figures given are of sections of the septa, so that his account does not form a good basis for the assigning of functions to the septal muscles. It seemed necessary, therefore, to re-examine the septa in the earthworm.

THE STRUCTURE OF THE SEPTA

The structure of the septa was examined by dissection under a binocular microscope of worms fully narcotized with 8% magnesium sulphate and killed by slow injection of 5% formaldehyde. This treatment left the worms extended and the septa well stretched.

The existence of a ventral perforation in each septum around the nerve cord was verified. This perforation is here termed the ventral foramen. Its upper boundary is clearly indicated by the paired ventroparietal vessels which run in it. Also, in the region of the testis sacs, where the ventral vessel parts company with the ventral nerve cord, to run dorsally to the testis sacs, an additional, but smaller, foramen exists in septa 9/10, 10/11 and 11/12 to allow of a space around the vessel.

The septa as far back as the 10th segment are stouter and more muscular than those from the 10th to the 19th segment, whilst septa 19/20, 20/21 and 21/22, which are attached to the posterior part of the gizzard, are particularly muscular, being the stoutest septa in the body. In addition to their intrinsic musculature the septa between all segments anterior to segment 8 are provided with slips of muscle which leave the posterior face on each side of the body and traverse the coelom before becoming attached to the lateral body wall. Septa anterior to that dividing segment 13 from 14 have, in addition, a short strand of muscle attached to each side of the ventral foramen and running posteriorly to become attached to the ventral body wall about half-way along the segment. In the region of the pharynx numerous strands of muscle, probably concerned with the retraction of the pharynx, run from it through the first three septa to join the muscles of the body wall. Despite these variations, the general structure of the septa is nearly constant and for the purposes of the present discussion it is sufficient to describe a typical septum.

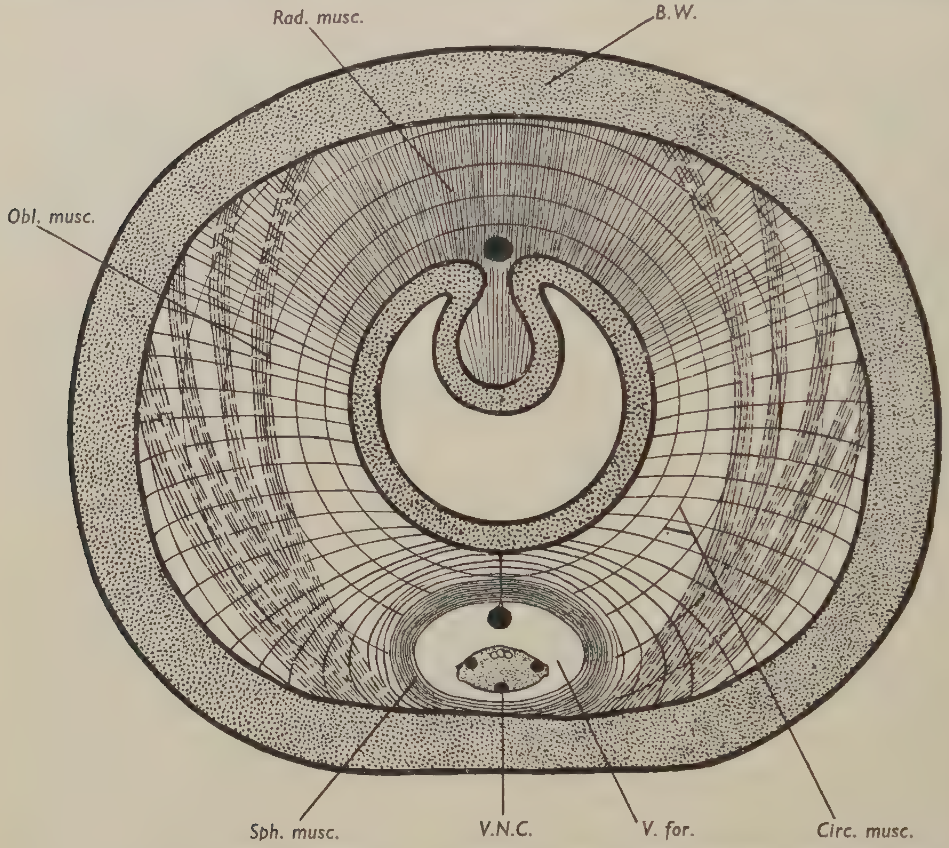

Fig. 1. Diagram to show the arrangement of the septal muscles: *B.W.*, body wall; *Circ. musc.*, circular muscles; *Obl. musc.*, oblique muscles; *Rad. musc.*, radial muscles; *Sph. musc.*, sphincter of ventral foramen; *V. for.*, ventral foramen; *V.N.C.*, ventral nerve cord.

The intrinsic musculature of the septa was examined partly in septa dissected out from the body and mounted as flat as possible on a slide, and partly from thick and thin microtome sections of complete worms. By these means it was verified that no perforation in the septum exists, apart from the ventral foramen and those allowing the passage of the ventral vessel in the region of the testis sacs.

The various sets of muscles which are arranged between the anterior and posterior layers of peritoneum are conveniently described as if they were viewed either from the front or rear of the septum, that is, as they would be seen in surface view of a stretched and flattened preparation. Four sets of muscles were recognized (Fig. 1).

(1) *Radial muscles*. Throughout the major part of each septum, strands of muscle run in a radial direction from the body wall to the gut. They are much more numerous in the dorsal part than elsewhere in the septum, gradually decreasing in number towards its ventral part and absent altogether from the neighbourhood of the ventral foramen. In the ventral parts of the septum the radial fibres depart from their truly radial orientation and curve ventrally towards the body wall so that they form a series of arcs from the ventral border of the gut to the ventro-lateral body wall.

(2) *Circular muscles*. Concentrically arranged series of circularly running fibres are found throughout the septum, but they are interrupted ventrally by the ventral foramen.

(3) *Sphincter muscle of the ventral foramen*. Surrounding the ventral foramen, except on its mid-ventral boundary, is a prominent band of muscle fibres which appears to be a specialized part of the system of radial muscles of the septum, since at the borders of this sphincter the fibres pass by almost imperceptible changes of direction into the series of radial fibres.

(4) *Oblique muscles*. On each side of the mid-line bundles of muscle fibres fan out from their attachments to the ventral body wall and pass obliquely dorsalwards to become attached to the dorso-lateral body wall.

Despite a statement by de Ribaucourt (1901) that the septal muscles take origin from the deeper layers of the longitudinal muscles of the body wall, the evidence derived from an examination of sections seemed to show that it is to the bed of circular body-wall muscles that they are connected.

FUNCTIONS OF THE SEPTAL MUSCLES

It will be apparent from the description of the septal musculature that the circular radial and oblique fibres would provide a means whereby the septum could remain taut whilst the diameter of the body is being varied by the reciprocal action of the circular and longitudinal muscles of the body wall. The radial muscle fibres, inserted as they are in the peritoneum of the gut, may also play a part in restricting the occlusion of the gut lumen by the positive pressure of the coelomic fluid. It also is evident that the oblique muscles of the septum, when in a state of contraction, will tend to produce a dorso-ventral flattening of the body.

Unfortunately, attempts to obtain direct experimental evidence of the action of the septal muscles by electrical stimulation failed, since the stimulus used always evoked a simultaneous contraction of the longitudinal muscles of the body wall. This causes the body to become shorter but fatter, stretches the septa and masks any contraction of their intrinsic musculature, if, indeed, it takes place at all.

The evidence regarding the action of the septal muscles during locomotion is, therefore, indirect.

It seems reasonable to assume that the septa in an earthworm would behave, during locomotion, in a manner essentially similar to those of small aquatic oligochaetes such as *Tubifex*. In this worm direct observation of the septa is rendered possible by the transparency of the body wall, and it can be seen that during locomotion the septa undergo considerable distortion as the coelomic pressure is varied from one region of the body to another. The septa, as would be expected, bulge into segments where the pressure is low but remain taut during all phases of contraction and relaxation of the body-wall muscles. Any tension in the septal muscles will obviously tend to prevent the transmission of changes of pressure from one segment to the next, an effect favouring the localization of pressure changes and allowing of differential pressures to be maintained in different parts of the body. This topic is referred to again in the next section (p. 118).

With regard to the sphincter of the ventral foramen, it seems clear that in active worms it is usually in a state of contraction and effectively closes the ventral foramen so that the segmentally arranged coelomic compartments are isolated one from the other in the sense that coelomic fluid cannot pass freely along the body. This isolation of the fluid in the successive compartments can be clearly demonstrated by the simple experiment of attempting to force fluid into the body of an active worm by means of a hypodermic syringe. It is found that it is virtually impossible to inject any quantity of fluid into the body until a force is applied sufficient to rupture the septa. Yet, in a fully narcotized worm, fluid can be freely injected and causes the whole body to become inflated even under a pressure as low as 15 cm. water.

In an attempt to confirm some of these observations a few coelomic compartments in selected regions of the body were injected with a suspension of bismuth oxycarbonate. The worms so treated were then photographed by means of X-rays, allowed to wriggle for a few minutes and again photographed. Although not good, the photographs were sufficiently clear to show that the suspension remained confined to those segments into which it was originally injected, thus indicating that during locomotion fluid does not pass between the coelomic compartments.

MEASUREMENTS OF PRESSURE IN THE COELOMIC FLUID

Compared with the lugworm the earthworm is a very active creature and the insertion of a hypodermic needle through the body wall evoked violent wriggling movements and usually completely upset the normal locomotory pattern. Nevertheless, it was found possible to make a series of readings of the internal pressures by means of a manometer filled with Hédon-Fleig's fluid and connected to the coelomic cavity by means of a hypodermic needle in the same way as was used to measure pressures in the lugworm (Chapman & Newell, 1947). The pressures recorded in this way were, within the limits of experimental error, the same as if the manometer were filled with water and at least indicate the order of pressures inside the worms. The results are given in Tables 1 and 2 whilst Table 3 is a record of pressures in worms fully narcotized with 8% magnesium sulphate.

The hydrostatic pressures in the coelomic fluid in various regions of the body of a fresh series of active worms was next measured by means of the glass spoon gauge apparatus set up and calibrated in essentially the same way as that described by

Table 1. *Pressures in cm. of water recorded by means of a capillary manometer at ½ min. intervals in segment 28 of active worms*

(Corrected for height of animal and capillary rise in the tube.)

Worm no.	0	1	2	3	4	5	6	7	8	9	10
1	23·5	13·0	13·5	worm slipped off needle							
2	24·0	19·0	18·0	17·0	15·0	15·0	15·0	14·0	13·5	12·5	13·0
3	23·0	19·5	19·5	19·0	19·0	19·0	18·0	17·0	18·0	17·0	17·0
4	22·0	20·0	20·0	20·0	20·0	18·0	17·0	16·0	17·0	17·0	17·0
5	22·0	20·5	23·0	23·0	19·0	17·0	16·5	16·5	16·5	16·0	16·0
6	22·0	20·0	20·0	20·0	16·0	16·0	16·5	16·5	16·0	16·0	16·0
7	22·0	17·0	14·0	14·0	13·5	15·0	16·0	18·0	18·5	17·0	17·0
8	22·0	22·5	23·5	23·5	13·5	16·5	17·0	18·5	19·5	21·0	22·0
9	38·0	22·0	19·0	19·0	13·0	16·0	13·5	14·0	13·5	13·5	14·0
10	25·0	22·0	20·0	20·0	17·0	17·5	17·0	16·5	15·5	16·0	16·0
11	25·0	21·5	21·0	21·0	20·0	17·0	17·0	15·5	16·0	15·5	15·0
12	25·0	20·0	20·0	20·0	17·0	15·5	15·0	13·5	14·5	16·0	16·5

Mean of initial pressures, 24·5 cm. water. Mean pressure in twelve worms, 17·5 cm. water. Mean of pressures after 5 min., 16·0 cm. water.

Table 2. *Pressures in cm. of water recorded by means of a capillary manometer at ½ min. intervals in tail region of active worms*

Worm no.	0	1	2	3	4	5	6	7	8	9	10
1	25·0	18·0	11·0	8·0	6·0	5·5	5·0	5·0	4·5	4·5	4·5
2	25·0	20·0	16·0	9·0	8·0	7·0	6·0	5·0	5·0	5·0	5·0
3	25·0	19·0	14·0	10·0	7·5	7·0	6·0	5·0	4·0	4·0	4·0
4	25·0	19·0	15·0	9·5	8·5	8·0	5·5	5·0	4·5	4·5	4·5
5	25·0	19·0	12·0	11·0	6·5	6·0	5·0	5·0	4·5	4·5	4·5
6	25·0	18·0	12·0	10·0	7·5	6·0	5·0	6·5	5·0	5·0	5·0

Mean pressure in six worms 8·0 cm. water.

Table 3. *Pressure in cm. of water recorded by means of a capillary manometer at ½ min. intervals in segment 28 of anaesthetized worms*

Worm no.	0	1	2	3	4	5	6	7	8	9	10
1	5·0	4·0	3·0	3·0	3·0	3·0	3·0	3·0	3·0	3·0	3·0
2	10·0	1·0	0·5	0	0	0	0	0	0	0	0
3	10·0	2·5	2·0	1·0	1·0	0	0	0	0	0	0
4	10·0	2·0	1·0	1·0	0·5	0	0	0	0	0	0
5	10·0	3·0	1·5	1·0	0·5	0	0	0	0	0	0
6	8·0	2·0	1·0	0·5	0	0	0	0	0	0	0

Except for worm no. 1, which revived slightly at the beginning of the experiment, pressures fell to zero at the end of 2½ min.

Picken (1936). A few trial readings sufficed to show that, as suspected, the measurements of coelomic pressures in the earthworm by means of a manometer is open to certain objections for, owing to the small amount of fluid in each coelomic compartment, an apparatus which will register differences in pressure under virtually isometric conditions is desirable. Also, it was apparent that the internal pressure changes vary rapidly so that a practically instantaneous recording is required. Spoon gauges satisfy both these conditions.

As in previous recordings, the apparatus was placed in communication with the body cavity of the worm by a hypodermic needle inserted through the body wall. The results of two series of readings are given in Tables 4 and 5.

Table 4. *Pressures in cm. of water recorded at ¼ and ½ min. intervals by means of a spoon gauge in the anterior third of active worms*

Series 1. Pressures recorded at ¼ min. intervals.

Worm no.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	25·0	23·0	16·0	8·0	19·0	8·0	16·0	16·0	13·0	18·0	20·0	Worm slipped off needle							
2	25·0	24·0	18·0	22·0	24·0	14·0	10·0	25·0	24·0	20·0	16·0	Worm slipped off needle							
3	25·0	25·0	14·0	25·0	14·0	8·0	10·0	14·0	16·0	11·0	29·0	8·0	5·0	18·0	16·0	Slipped off needle			
4	25·0	25·0	22·0	14·0	10·0	18·0	19·0	17·0	12·0	17·0	10·0	13·0	14·0	18·0	22·0	16·0	14·0	16·0	10·0
5	25·0	22·0	10·0	18·0	12·0	16·0	14·0	14·0	17·0	11·0	18·0	5·0	10·0	8·0	16·0	10·0	8·0	12·0	16·0
6	25·0	24·0	16·0	10·0	8·0	15·0	20·0	10·0	8·0	14·0	18·0	11·0	8·0	11·0	12·0	10·0	16·0	10·0	8·0

Mean pressure in six worms, 15·0 cm. water.

Series 2. Pressures recorded at ½ min. intervals.

Worm no.	0	1	2	3	4	5	6	7	8	9	10
1	27·0	7·0	Worm slipped off needle								
2	27·0	15·0	10·0	10·0	12·0	12·0	12·0	13·0	7·0	2·0	5·0
3	27·0	2·0	2·0	2·0	3·0	3·0	5·0	2·0	5·0	5·0	3·0
4	30·0	10·0	12·0	12·0	14·0	15·0	16·0	17·0	17·0	16·0	16·0
5	35·0	18·0	7·0	22·0	12·0	16·0	14·0	16·0	17·0	17·0	16·0
6	35·0	7·0	8·0	11·0	12·0	12·0	13·0	13·0	5·0	5·0	5·0
7	35·0	2·0	3·0	2·0	3·0	3·0	5·0	4·0	5·0	5·0	5·0
8	35·0	10·0	10·0	9·0	9·0	12·0	14·0	14·0	30·0	25·0	25·0
9	35·0	9·0	25·0	25·0	25·0	16·0	12·0	12·0	12·0	15·0	12·0
10	35·0	12·0	22·0	22·0	18·0	24·0	16·0	18·0	22·0	20·0	20·0

Mean pressure in nine worms, 12·0 cm. water. Worms nos. 3 and 7 appeared very sluggish. Average pressure from series 1 and 2, 13·5 cm. water.

Table 5. *Pressures in cm. of water recorded at ¼ and ½ min. intervals by means of a spoon gauge in the tail region of active worms*

Series 1. Pressures recorded at ¼ min. intervals.

Worm no.	0	1	2	3	4	5	6	7	8	9	10	11	12
1	25·0	14·0	14·0	8·0	10·0	14·0	16·0	11·0	19·0	8·0	5·0	14·0	10·0
2	25·0	14·0	10·0	14·0	7·0	10·0	7·0	7·0	9·0	12·0	6·0	8.0	8·0
3	25·0	16·0	11·0	8·0	7·0	10·0	8·0	16·0	6·0	6·0	8·0	6·0	6·0

Mean pressure in three worms, 10·0 cm. water.

Series 2. Pressures recorded at ½ min. intervals.

Worm no.	0	1	2	3	4	5	6	7	8	9	10	11	12
1	25·0	16·0	8·0	7·0	7·0	9·0	6·0	5·0	4·0	4·0	5·0	4·0	4·0
2	25·0	10·0	7·0	6·0	7·0	8·0	7·0	7·0	7·0	7·0	6·0	6·0	6·0
3	25·0	11·0	8·0	9·0	8·0	8·0	6·0	7·0	8·0	4·0	4·0	7·0	7·0

Mean pressure in three worms, 7·0 cm. water. Average from series 1 and 2, 8·5 cm. water.

DISCUSSION OF RESULTS

From Tables 4 and 5 it will be seen that in the anterior third of the body of an actively wriggling earthworm there is an average hydrostatic pressure of about 13·5 cm. water, but that this pressure is subject to considerable and fairly rapid changes, falling as low as 2·0 cm. water and rising as high as 29 cm. water. The figures for the tail end of active worms are lower, the mean pressure being 8·5 cm. water and the fluctuation being from 4 to 19 cm. water. The pressures measured in the second series of worms were lower than in the first series, and this result is probably due to the worms being less active.

The mean pressures recorded both for the anterior and tail regions of the body are in good agreement with those measured by means of the capillary manometer, but show more clearly how the pressure fluctuates. The manometer readings showed a gradual fall over a period of time with practically no fluctuations, and whilst it is true that, on the whole, the readings taken with the spoon gauge were also lower towards the ends of the series and that the readings did not extend over such a long period of time, the significant feature is the recording of large fluctuations of pressure.

It was found difficult to correlate in any precise way the changes in pressure with definite muscular configurations, but it was noted that the highest pressures occurred when the circular muscles were fully contracted. A state of moderate relaxation of both sets of body-wall muscles is associated with low hydrostatic pressures whilst, as is shown in Table 3, the pressure in narcotized worms is zero.

The general fall in pressure towards the ends of the series of recordings may, perhaps, be accounted for by the fatigue of the body-wall muscles, whilst the lower mean obtained from the measurements with the spoon gauge when compared with manometric measurements seems to be due to the fluctuations. It seems certain that the pressure in worms connected to a manometer is steadied by fluid entering the body cavity from the manometer, and also because the amount of fluid in any one coelomic compartment is small when compared with the volume in the apparatus. Variations in pressure are therefore smoothed out in a way not possible with a practically isometric apparatus like the spoon gauge.

THE FORCES DEVELOPED DURING LOCOMOTION

It seems fair to assume that, as in the lugworm, the forces available to an earthworm for developing a forward thrust and for causing changes in the shape of the body are brought about by the interaction of the body-wall muscles and coelomic fluid, and that an estimate of these forces can be arrived at indirectly by calculation from the pressures recorded. The pressure in the coelomic fluid will be distributed uniformly, and when the circular muscles of the body wall contract the body will become extended with a force proportional to the pressure and to the cross-sectional area in the region of the body under consideration. Conversely, when the longitudinal muscles contract and the circulars relax, the diameter of the body will be increased by the pressure making itself apparent by forces acting radially. It is clear that since the septa do not behave as rigid partitions the pressure in any one segment cannot exceed that in a neighbouring segment without causing the septum to bulge. Any tension in the septal muscles will, however, cause resistance to bulging, and the cumulative effect of the resistance of several septa is appreciable and allows of pressure differences to be set up and maintained in different parts of the body. This effect was tested by connecting a spoon gauge to the coelom in the anterior third of the body so that pressure could be measured whilst, at the same time, saline solution was forced into the coelom in the clitellar region by means of a hypodermic syringe. It was found that the pressure in the anterior third rose only very slightly (by about 1 cm. water, in fact) until the pressure set up by the syringe was sufficient to rupture the septa and to drive fluid forwards into the segments in which pressures were being measured.

It has already been demonstrated that the septa divide the body of an earthworm into functional fluid-tight compartments so that alterations in the shape of the body must be brought about solely by the contractions of the individual segments and are not, as in some worms, accompanied by movements of fluid from one part of the body to another. Further, any thrust exerted by the extreme anterior end of the worm when the body is elongating, must be equal to the total force applied by the coelomic fluid to the first septum (i.e. that separating segment 4 from segment 5), the first four segments, in which the body cavity is virtually occluded, acting as a solid muscular organ. In the worms examined, the highest pressure recorded in the anterior third of the body was 29 cm. water and, neglecting the two sluggish specimens, the lowest was 5 cm. water. An average figure for the diameter of the body in the region of the first septum is about 0·7 cm. when the circular muscles are fully relaxed. In this region of the body the gut has a diameter of about 0·35 cm. so that the area of the first septum is

$$3{\cdot}14 \times 0{\cdot}35^2 - 3{\cdot}14 \times 0{\cdot}17^2 = 0{\cdot}295 \text{ sq.cm.}$$

The force acting on the septum causing it to bulge forwards and transmit pressure to the segments in front was, in this instance, equal to $29 \times 0{\cdot}295 = 8{\cdot}5$ g. It is of interest to note that this figure, arrived at very indirectly, agrees well with the upper figure given by Gray & Lissmann (1938*b*), who measured the forces by means of a special torsion balance. Gray & Lissmann gave the range of forces as from 2 to 8 g., and from the lowest figure for pressures in the anterior third of the body, namely, 5 cm. water, a thrust of 1.5 g. can be calculated which again is a good agreement with the figures obtained by direct measurement.

Forces of this order probably suffice to allow earthworms to burrow into and through soils of varying resistance, even quite heavy clays, but of importance in forcing a way through the soil is the actual pressure which can be applied by the conical front end of the body. If, for example, the diameter of the prostomium which forms the apex of a cone, the rest of which is formed by the first four segments, is 1·0 mm., then its area is 0·008 sq.cm. Assuming as before that the force applied to the first septum is equivalent to 8·5 g., then the pressure of the prostomium against the soil will be $8{\cdot}5/0{\cdot}008 = 1060$ g./sq.cm.

When burrowing, the pointed anterior end of the body is forced into any crevices between the soil particles, and the gap is then widened by inflation of that part of the body which has entered below the surface. Pressures have not been recorded during this phase of activity but are probably much higher than any obtained during wriggling, since they would be due to the contraction of the powerful longitudinal muscles of the body wall which, as can be seen in cross-section, are far more bulky than the circular muscles. When the worm is on the surface the longitudinal musculature cannot, of course, generate a pressure in the coelomic fluid greater than can be withstood by the circular muscles of the body wall. On the other hand, when a worm is in its burrow the body wall will be prevented from undue expansion or bursting by the walls of the burrow, and this may allow the longitudinal muscles to raise the pressure in the coelom to much higher levels than have been recorded in this investigation. Penetration of the anterior end of the body into the soil is often accompanied by a to-and-fro rotation of the anterior end of the body in much the same way as a bradawl is used for boring into wood. This movement aids in the loosening of soil particles. Then, once a portion of the body has entered the soil the worm can get a grip on the sides of the burrow and the rest of the body can be drawn inwards and the burrow be gradually extended.

It will be seen that a watertight septate construction of the body is favourable to the mechanism of crawling as described by Gray & Lissmann (1938*a*) in which an essential feature is the contraction of the longitudinal muscles of a few segments to form a 'foot' or *point d'appui* which resists the reaction to a forward thrust by the anterior end of the body and also the reaction to the tension which drags forward the posterior end of the body of the worm.

Chapman (1949)* points out that in a cylindrical, fluid-filled animal the constriction of the circular muscles at one end would produce an effect on the shape of the body dependent on the tensions in the circular and longitudinal muscles in other regions, and only if the pressures exerted by these latter muscles on the coelomic fluid exceeds that exerted by the longitudinal muscles of the constricting region, would that end necessarily elongate.

On the other hand, if the body is divided into separate regions by septa which can actively resist the pressures produced by contraction of the body-wall muscles, then contraction of the circular muscles may produce effects which can be confined to particular regions of the body. For example, in a crawling earthworm the contraction of the longitudinal muscles in the region of the *point d'appui* would have the effect of stretching the septa since they are watertight and the diameter of the body will increase. In the region anterior to the *point d'appui*, which is elongating because of the contraction of the circular muscles, the pressure can be high (since only by an increased pressure can the contraction of the circular muscles be translated into movement). On the other hand, the pressure in the region posterior to the *point d'appui* need only be low, since the tail is being passively dragged forward by the contraction of the longitudinal muscles for whose action no intervention of a fluid transmission system is necessary. Were it not for the septate construction of the body it is difficult to see how it would be possible simultaneously to extend the anterior end by increased pressure and to retract the posterior end at reduced pressure.

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* Private communication.

THE MECHANISM FOR THE PREVENTION OF LEAKAGE OF COELOMIC FLUID

To any animal whose locomotion and turgidity depends on a body-wall muscle-body fluid system and which may be said therefore to possess a hydrostatic skeletal system, it is clearly essential that there shall be no uncontrolled escape of fluid through any pores or ducts which lead from the coelom to the exterior. This is particularly true of a terrestrial animal such as the earthworm which is frequently faced with the danger of desiccation by mere evaporation from the surface of the body. It is not surprising to find, therefore, that the nephridiopores and dorsal pores are provided with sphincters which effectively control the outward passage of coelomic fluid under the range of pressures normally found in active worms. Even in the lugworm the nephridiopores open only rather rarely to allow of voiding of the urine—every 5 sec. according to Strunk (1930)—whilst an old observation of Cuénot (1898) gives the rate of opening of the nephriodipores of the earthworm as once every 3 days. This has not, apparently, been confirmed, but a smaller quantity of urine is to be expected in a land animal where conservation of water is often of importance.

In order to test at what pressure leakage of the coelomic fluid occurs, a worm was bisected and a canula was then inserted at the cut end to one side of the gut. A ligature around the body and canula sufficed to make a pressure-tight joint. The canula was then connected to a manometer filled with Hédon-Fleig's fluid by which means the pressure was increased by 10 cm. of water at a time and allowed to remain at each pressure for 1½ min. to allow the apertures to be observed for leakage. No leakage was observed even when the pressure was raised to 150 cm. of water which is well above the normal working range.

A pressure of a mere 20 cm. of water is, however, sufficient to cause water to spurt from the dorsal pores of a narcotized worm. Even in narcotized specimens, however, the nephridiopores and gonoducts did not leak under high pressures, since their internal apertures seemed to act as valves which close when the internal pressure rises.

SUMMARY

1. A short review is given of the coelom and of its morphological relations in the earthworm.

2. The arrangement of the intrinsic muscles in a typical septum is described. Four main sets of muscles are recognized: viz. radial muscles, circular muscles, oblique muscles, and the sphincter around the ventral foramen.

3. It is suggested that the function of the radial, circular and possibly of the oblique muscles is to control bulging of the septa, and so serve to localize differences in pressure in the coelomic fluid. Normally, in active worms, the sphincter of the ventral foramen is contracted and forms an effective barrier to the passage of fluid

from one coelomic compartment to the next. This was verified experimentally and by X-ray photography.

4. A series of measurements of the pressure in the coelomic fluid in different regions of active worms was recorded by means of a capillary manometer and by the use of a spoon-gauge apparatus. Manometric measurements showed the average pressure in the anterior third of the body to be 16·0 cm. water and in the tail region to be 8·0 cm. water. The corresponding figures obtained with a spoon gauge were 13·5 and 8·5 cm. water. The pressure in narcotized worms is zero.

5. These results are discussed, and it is pointed out that the manometer readings suffer from the disadvantage of failing to show the rapid fluctuations in pressure which occur during wriggling movements of the worms.

6. It is calculated from these pressure readings that a worm can exert a forward thrust equivalent to forces of between 1·5 and 8·0 g. These figures agree well with those obtained by Gray & Lissman by the use of a special torsion balance.

7. The burrowing movements of earthworms are briefly described.

8. It is shown that the sphincters of the dorsal pores and of the nephridiopores do not normally allow of the escape of coelomic fluid, and will withstand a pressure well outside the normal range.

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